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(54) Title: IMPROVEMENTS IN THE EXPRESSION OF NEWLY INTRODUCED GENES IN YEAST CELLS		
(57) Abstract The invention relates to an improved expression system for newly introduced genes in yeast and comprises a yeast regulon, and preferably a transcription terminator, derived from one of the GAPDH genes of <i>S. cerevisiae</i> . The about 850 nucleotides long GAPDH regulon described proved to be almost ten times as effective as smaller regulons described previously. Said regulon and/or terminator can be introduced into yeasts either as part of plasmide molecules or by incorporating into the yeast genome. Vectors containing the expression system preferably comprises an autonomously replicating sequence derived from <i>K. lactis</i> (KARS) or an origin of replication originating from the <i>S. cerevisiae</i> 2 micron yeast plasmid. After transformation of yeasts, in particular of the genera <i>Kluyveromyces</i> and <i>Saccharomyces</i> , with said vectors, the yeasts transformed can produce foreign proteins more effectively. In the case of thaumatin the presence of a signal polypeptide (the pre-part) appears to be essential for expression in yeast. DNA sequences encoding other signal polypeptides are also described. The use of codons preferred by yeast in both structural genes, preferably those encoding thaumatin-like proteins and chymosin-like proteins, and DNA sequences encoding signal polypeptides is also described.		

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IMPROVEMENTS IN THE EXPRESSION OF NEWLY INTRODUCED
GENES IN YEAST CELLS

The present invention relates to improvements in the expression of newly introduced genes in yeast cells.

In particular the invention relates to a DNA sequence capable of initiating transcription by yeast RNA polymerase II which includes at least part of the regulon region of one of the GAPDH genes of S. cerevisiae.
(DNA = DeoxyriboNucleic Acid; RNA = RiboNucleic Acid; GAPDH = GlycerAldehyde-3-Phosphate DeHydrogenase; mRNA = messenger RNA).

The invention further relates to the use of a DNA sequence capable of both termination of the transcription by yeast RNA polymerase II and effecting polyadenylation of the mRNA, which includes at least part of the termination/polyadenylation region belonging to one of the GAPDH genes of S. cerevisiae.

The invention also relates to a larger rDNA sequence which contains at least the above-indicated regulon region of one of the GAPDH genes of S. cerevisiae and one or more structural genes different from the GAPDH genes of S. cerevisiae, which DNA sequence can be inserted into a recombinant DNA plasmid or into a yeast chromosome in order to transform yeasts so that they become able to produce the desired proteins encoded by the structural genes.

Finally, the invention relates to a process for preparing a protein by cultivating a yeast containing the above-mentioned larger rDNA sequences under conditions whereby the protein is formed and isolating the protein from that yeast culture, as well as the proteins produced by such a process.



BACKGROUND OF THE INVENTION

Developments in recombinant DNA (= rDNA) technology have made it possible to isolate or synthesize specific genes or portions thereof from higher organisms such as man, animals and plants, and to transfer these genes or gene fragments to microorganisms such as bacteria or yeasts. The transferred gene is replicated and propagated as the transformed microorganism replicates. As a result, the transformed microorganism may become endowed with the capacity to make whatever protein the transferred gene or gene fragment encodes whether it is an enzyme, a hormone, an antigen, an antibody, or a portion thereof. The microorganism passes on this capability to its progeny, so that in effect, the transfer has resulted in a new microbial strain, having the described capability.

A basic fact underlying the application of this technology for practical purposes is that DNA of all living organisms, from microbes to man, is chemically similar, being composed of the same four nucleotides. For example, the same nucleotide sequence which codes for the amino acid sequence specifying preprochymosin in stomach cells of newborn mammals, will, when transferred to a microorganism, be recognized as coding for the same amino acid sequence.

The basic constituents of the recombinant DNA technology are formed by:

- i) the gene encoding the protein of interest.
- ii) a vector (plasmid) in which the new gene has to be inserted to guarantee stable replication and a high level of expression of the gene.
- iii) a suitable host microorganism in which the vector carrying the new gene can be introduced.



- Depending on the nature of the protein to be synthesized, the industrial application of this protein and the technically possible fermentation and purification procedures, the plasmid vector and the host organism have to be selected. In most cases the selection of a host organism which is unsuspected with regard to the production of toxic substances, i.e. a microorganism mentioned in the GRAS (Generally Recognized As Safe) list, will be highly important. However, only very few of these GRAS-microorganisms meet all requirements asked for by the application of either the recombinant DNA or the fermentation technology. A selection on the basis of these positive criteria shows that, at this moment, certain yeast species, notably *Saccharomyces*, *Kluyveromyces*, *Debaryomyces*, *Hansenula*, *Candida*, *Torulopsis* and *Rhodotorula*, can be regarded as very promising host organisms for genetically engineered DNA molecules.
- In the present invention use is made of recombinant DNA, molecular, biological and chemical techniques to construct plasmid vectors that can be stably maintained within yeasts and, most importantly, contain the appropriate regulons to bring about a high level of expression of newly introduced genes.

- Several plasmid vectors are known nowadays which can be used for the transformation of the yeast *Saccharomyces cerevisiae* [C.P.Hollenberg, Current Topics in Microb. and Immunol. 96, 119-144 (1982) and A. Hinnen and B. Meyhack, Current Topics in Microb. and Immunol. 96, 101-117 (1982)]. These vectors rely on either autonomous replication sequences (ARS) isolated from the chromosomal DNA of particular yeast species or the replicating ability of the 2 micron DNA plasmid found in *Saccharomyces cerevisiae* to maintain the vector and the inserted gene within the host cell. Additionally



these yeast vectors contain a marker by which transformants can be selected. Examples of such markers are the leu 2 gene [A. Hinnen et al., Proc.Natl.Acad.Sci. USA, 75, 1929-1933(1978)], the trp 1 gene [K. Struhl et al., Proc.Natl.Acad.Sci.USA, 76, 1035-1039(1979)], the lactase gene [R.C. Dickson, Gene,10, 347-356 (1980)], and genes which confer resistance of the host cell against certain antibiotics.

- 10 The stability of AR-sequences in S. cerevisiae or (K)AR sequences in Kluyveromyces lactis or K. fragilis is not always sufficient for the development of a reliable fermentation process using these yeasts. Therefore, integration of the foreign structural gene into the
- 15 chromosome(s) of the new host cell can be very important for the industrial application of rDNA-containing yeasts.

Seen from an economic point of view not only the

20 stability of the inserted gene within the yeast cell is important but also the efficiency with which this gene is expressed as protein. Based on today's knowledge, the main routes to achieve high levels of expression of a newly inserted gene include:

- 25
- integration of the structural gene downstream of a promoter (RNA-initiation) site which can effect a high transcription frequency of the gene. Ideal is that the promoter activity is inducible, i.e. can be
- 30 switched on or off depending upon a temperature shift or the presence of an inducer in the growth medium. Potent promoters operating in yeasts are those responsible for transcription of the genes encoding glycolytic enzymes. Experimental work done by Maitra
- 35 and Lobo [J.Biol.Chem., 246, 489-499 (1971)] suggests furthermore that some of these promoters are highly inducible. However a serious difficulty with regard



to the isolation of such promoters is that up to now little is known about the nucleotide sequences which confer regulation and full promoter activity on the DNA fragment

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- integration of an RNA (RiboNucleic Acid) termination signal downstream of the structural gene. Owing to the presence of such a termination signal, transcription of the structural gene cannot interfere with the transcription of adjacent operons. Moreover transcription seems to be more efficient [K.S. Zaret and F. Sherman, Cell, 28, 563-573 (1982)] and polyadenylation of the messenger is likely to occur correctly, resulting in a more stable mRNA (messenger RNA) population. However, up to now exact data on nucleotide sequences required for termination of transcription in yeasts are not available.

- the presence of a nucleotide sequence flanking the AUG codon in the RNA-molecule which is optimal for protein synthesis initiation. According to published data [M. Kozak, Nucleic Acids Res. 9, 5233-5252 (1981)] the positions -3 and +4, N X X A U G N are highly conserved (A or C at -3 and G at +4), which observation suggests a role for these nucleotides in the recognition of the AUG codon as a translation start point by the ribosome. Although one might expect an efficient yeast promoter to contain either an A or C as nucleotide at the -3 position, the nucleotide at the +4 position forms part of the coding sequence and is therefore dependent upon the nature of the gene to be inserted downstream of the promoter. This indicates that it will be difficult to fulfil this condition in all cases.

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- the copy number of the vector within the host cell. In most cases high copy numbers will lead to higher



mRNA levels and, therefore, to higher expression levels of a gene. In S. cerevisiae vectors containing the 2 micron DNA replication origin can reach copy-numbers as high as 50. This is considerably more than can be reached by for instance integrating vectors or vectors containing autonomously replicating sequences (ARS) [K. Struhl et al., Proc.Natl.Acad.Sci.USA, 76, 1035-1039 (1979) and A.J. Kingsman et al., Gene, 7, 141-152 (1979)]. However, in yeast species other than Saccharomyces, 2 micron DNA has not yet been found, suggesting that its replication origin will be functional in a very limited number of yeast species only. Experimental data obtained so far show that the 2 micron replication origin is functional in Schizosaccharomyces pombe [D. Beach and P. Nurse, Nature, 290, 140-142 (1981)], but not in Kluyveromyces lactis (G. Das and C.P. Hollenberg, Curr. Genet. 5, 123-128 (1982)].

Therefore the transformation of yeasts belonging to genera other than Saccharomyces or Schizosaccharomyces will be dependent in most cases upon the availability of other DNA replication origins such as ARS isolated from the organism to be transformed or upon the integration of the foreign gene into the yeast genome.

- a codon use of the gene which is optimal for the host organism used. Results obtained with the yeast S. cerevisiae show a strong correlation between the abundance of certain tRNA (transfer RNA) species and the occurrence of the respective codons in its protein genes. Therefore, optimal expression of for instance the bovine preprochymosin gene or the plant preprothaumatin gene in S. cerevisiae would require a chemical synthesis of both genes with a codon population which correlates with these abundant yeast tRNA species.

- an additional factor which might influence the translation of a gene is the presence of a DNA sequence encoding a so-called signal sequence. These signal sequences are in most cases hydrophobic N-terminal protein extensions which are often involved in the process of cotranslational secretion of the protein through a membrane. In the present specification new data are shown obtained with the expression of the preprothaumatin gene in yeast, indicating that when the DNA sequence encoding the signal protein is removed from the gene, expression of the gene is reduced by a few orders of magnitude.

At present the number of known yeast species which can serve as a host for recombinant DNA molecules is limited to Saccharomyces cerevisiae and Schizosaccharomyces pombe.

SUMMARY OF THE INVENTION

Therefore, a need exists for additional yeast species with other metabolic properties and other nutritional demands, so that the field to which recombinant DNA technology can be applied on a commercial level will be broadened. However, the use of new yeast species requires suitable DNA replication origins to guarantee a stable replication of the vector molecule in the new host as well as an appropriate expression system for newly inserted DNA. The present invention provides an expression system, the use of which is not restricted to S. cerevisiae but which can function in other yeast species as well. In order to achieve this the RNA initiation/regulation and the RNA termination/polyadenylation signals of a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene of S. cerevisiae were isolated.



Besides the fact that the RNA initiation site of a GAPDH gene is a very efficient promoter in S. cerevisiae [M.J. Holland et al. Biochemistry 17, 4900-4907 (1978)] the promoter according to the present invention was isolated and applied after it was realized that GAPDH is a metabolic key enzyme in many different organisms, suggesting a certain conservatism during evolution with regard to the nucleotide sequence regulating its expression. On this basis further experiments were carried out. It was observed (i) that the isolated and radioactively labelled GAPDH regulon fragment hybridized with colonies of various yeast species, (ii) the S. cerevisiae GAPDH regulon expressed foreign genes in K. lactis efficiently and (iii) the larger regulon region of about 850 nucleotides was more effective than the smaller regulon region of about 280 nucleotides published by Holland J.P. and Holland M.J. in J.Biol.Chem. 255, 2596-2605 (1980). These new findings gave us confidence that the regulon isolated will prove to be useful in the expression of foreign DNA in a number of other yeast species.

The present invention provides a DNA sequence capable of initiating transcription by yeast RNA polymerase II which includes at least part of the regulon region of one of the GAPDH genes of S. cerevisiae, characterized in that it comprises a DNA sequence essentially as given in Fig. 2, and wherein the regulon region is optionally modified to include at least one restriction enzyme cleavage site, to facilitate manipulation of the nucleotide sequence region for protein synthesis initiation.

An example of a modification of the regulon is given in item 4 and Figs. 7A and 8, where the introduction of a Sac I site is described. Although in Fig. 2 only one specific DNA sequence is described, it will be clear to



the expert that modifications of this DNA sequence, either by replacement of one or more nucleotides or by addition or deletion of one or more nucleotides, which modifications do not impair the properties of the regulon region given in Fig. 2, are within the realm of the invention.

The invention further provides a DNA sequence capable of both termination of the transcription by yeast RNA polymerase II and effecting polyadenylation of the mRNA, which includes at least part of the termination/polyadenylation region belonging to one of the GAPDH genes of S. cerevisiae, characterised in that it comprises a DNA sequence essentially as given in Fig. 3.

Indications have been obtained that the presence of such DNA sequence is favourable for the expression of structural genes in yeasts. The presence of such DNA sequence seems to be particularly advantageous for incorporating the rDNA in the genome of a yeast cell.

The invention also provides a DNA sequence, which can be inserted into a recombinant DNA plasmid or into a yeast chromosome, comprising:

- (a) a DNA sequence essentially as given in Fig. 2, and
- (b) one or more structural genes different from the GAPDH genes of S. cerevisiae, and at least two of features (c)-(f),
- (c) one or more specific DNA sequences capable of terminating the transcription by yeast RNA polymerase II and effecting polyadenylation of the mRNA, and/or
- (d) one or more selection markers, and/or
- (e) either one or more nucleotide sequences allowing a stable insertion in a chromosome of yeasts or one or more DNA sequences which regulate DNA replication in yeasts belonging to the genus Saccharo-



myces or to the genus Kluyveromyces, and/or

- (f) a DNA sequence encoding a signal polypeptide of not more than 30 amino acid residues assisting the translocation of proteins, which DNA sequence is situated upstream of and in the same reading frame as the structural gene.

In particular, the structural gene of (b) encodes thaumatin or chymosin, or their various allelic or modified forms, which modified forms do not impair the sweet-tasting properties of thaumatin or the milk-clotting properties of chymosin, respectively, or precursors of these thaumatin-like or chymosin-like proteins, since such DNA sequence will assist in giving an increased expression of these genes.

A preference exists to use a DNA sequence essentially as given in Fig. 3 as the specific DNA sequence of (c), since this seems more adapted to yeast than other termination/polyadenylation regions.

The DNA sequence of (f) encoding a signal polypeptide can be selected from the group consisting of DNA sequences encoding

- (a) the signal polypeptide translocating S. cerevisiae invertase, namely Met.Leu.Leu.Gln.Ala.Phe.Leu.Phe.-Leu.Leu.Ala.Gly.Phe.Ala.Ala.Lys.Ile.Ser.Ala;
- (b) the signal polypeptide translocating S. cerevisiae acid phosphatase, namely Met.Phe.Lys.Ser.Val.Val.-Tyr.Ser.Ile.Leu.Ala.Ala.Ser.Leu.Ala.Asn.Ala;
- (c) the signal polypeptide of unmaturred forms, of thaumatin-like proteins, namely Met.Ala.Ala.Thr.-Thr.Cys.Phe.Phe.Phe.Leu.Phe.Pro.Phe.Leu.Leu.Leu.-Leu.Thr.Leu.Ser.Arg.Ala;
- (d) the signal polypeptide of unmaturred forms of chymosin-like proteins, namely Met.Arg.Cys.Leu.Val.-Val.Leu.Leu.Ala.Val.Phe.Ala.Leu.Ser.Gln.Gly; and



(e) two consensus signal polypeptides, namely
 Met.Ser.Lys.Ala.Ala.Leu.Ala.Phe.Ile.Ala.Phe.Val.-
 Ile.Val.Leu.Ile.Val.Asn.Ala and
 Met.Ser.Lys.Phe.Val.Ile.Val.Leu.Ile.Val.Ala.Ala.-
 5 Leu.Ala.Phe.Ile.Ala.Asn.Ala.

In order to make the conditions for expression as good
 as possible it is advocated to modify the structural
 gene of (b) and/or the signal polypeptide-encoding DNA
 10 sequence of (f) such that the codons are codons pre-
 ferred by yeasts. Following the teaching of J.P.
 Holland and M.J. Holland (J.Biol.Chem. 255, 2596-2605
 [1980]) the preferred codons are:

	GCC or GCT	alanine	TTG	leucine
15	AGA	arginine	AAG	lysine
	AAC	asparagine	ATG	methionine
	GAC or GAT	aspartic acid	TTC	phenylalanine
	TGT	cysteine	CCA	proline
	CAA	glutamine	TCC or TCT	serine
20	GAA	glutamic acid	ACC or ACT	threonine
	GGT	glycine	TGG	tryptophan
	CAC	histidine	TAC	tyrosine
	ATC or ATT	isoleucine	GTC or GTT	valine.

25 The DNA sequences described above are not a purpose in
 themselves. They will be used in a process for pre-
 paring yeasts containing these DNA sequences by intro-
 ducing these rDNA sequences into Saccharomyces,
Kluyveromyces, Debaryomyces, Hansenula, Candida,
 30 Torulopsis or Rhodotorula yeasts, either in the form of
 plasmids or by incorporation in the yeast genome.

The invention further provides yeasts containing such
 rDNA sequences, either in the form of a plasmid or in-
 35 corporated in the yeast genome and their use in a pro-
 cess for preparing a protein by cultivation of such
 yeast, whereby the rDNA sequence incorporated in the



yeast contains a structural gene encoding that protein or a precursor thereof, which precursor can form the relevant protein during processing.

- 5 Finally, the invention provides the proteins produced by this novel process.

Several constructions in which the GAPDH promoter/
regulation region was combined with structural genes
10 encoding either preprothaumatin or preprochymosin or
some of the various maturation products have been made
and synthesis of thaumatin-like proteins in yeast have
been demonstrated. Preferred constructions contained
structural genes encoding either preprothaumatin or
15 prethaumatin. To improve the expression yield of said
genes, the original codons can be replaced by codons
which are abundantly present in highly expressed genes.
Moreover, the DNA sequence encoding the signal sequence
of preprochymosin can be replaced by DNA sequences en-
20 coding signal sequences of products excreted by yeasts,
such as the signal sequences invertase and acid phos-
phatase produced by S. cerevisiae.

The DNA sequences according to the invention may com-
25 prise nucleotide sequences which regulate DNA repli-
cation in yeasts belonging to the genera Saccharomyces
and Kluyveromyces. If the replication origin is in-
serted into an rDNA plasmid, the following combinations
are preferred for Saccharomyces: a combination of the
30 replication origin of the 2 micron DNA with the leu 2
gene as is present on plasmid pMP81 [C.P. Hollenberg,
Current Topics in Microbiol. and Immunol. 96, 119-144
(1982)] and a combination of the replication origin of
the 2 micron DNA in combination with the trp 1 gene
35 present on plasmid YRp7 [D.T. Stinchcomb et al., Nature
282, 39-43 (1979)]. A preferred replication origin for
Kluyveromyces consists of the KARS-2 sequence in com-



ination with the trp 1 gene as is present on plasmid pEK 2-7 described in European Patent Application N° 0096430(A1) on pages 21 and 25 and in Fig. 2

If the DNA sequence has to be inserted into a yeast genome, it is preferable that the DNA sequence contains the termination region of Fig. 3 downstream of the structural gene besides the regulon region of Fig. 2 upstream of the foreign structural gene, which combination will be inserted by homologous recombination at the position of the GAPDH gene in the yeast genome (cf. Fig. 19). An alternative may be that the combination regulon region - structural gene - termination region is inserted into a cloned DNA sequence derived from yeast genome (K. Struhl, Nature 305, 391-397 [1983] and R.J. Rothstein, Methods in Enzymology 101, 202-211 [1983]).

For a better understanding of the invention the most important terms used in the description will be defined:

An operon is a gene comprising (a) a particular DNA sequence [structural gene(s)] for polypeptide(s) expression, (b) a control region or regulon (regulating said expression) upstream of the structural gene and mostly consisting of a promoter regulation sequence, (c) a ribosome binding- or interaction DNA sequence and (d) a control region or transcription terminator downstream of the structural gene.

Structural genes are DNA sequences which encode through a template (mRNA) a sequence of amino acids characteristic for a specific polypeptide.

A promoter is a DNA sequence within the regulon to which RNA polymerase binds for the initiation of the transcription.

A terminator is a DNA sequence within the operon



comprising amongst others particular DNA sequences involved in the polyadenylation of mRNA and particular DNA sequences involved in the termination of the transcription of DNA by RNA-polymerase.

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Reading frame. The grouping of triplets of nucleotides (codons) into such a frame that at mRNA level a proper translation of the codons into the polypeptide takes place.

10

Transcription. The process of producing mRNA from a structural gene.

15

Translation. The process of producing a polypeptide from mRNA.

20

Expression. The process undergone by a structural gene to produce a polypeptide. It is a combination of many processes, including at least transcription and translation.

25

By signal sequence (also called signal polypeptide or leader sequence) is meant that part of the pre(pro)-protein which has a high affinity to biomembranes or special receptor-proteins in biomembranes and/or which is involved in the transport/translocation of pre(pro)-protein. These transport/translocation processes are often accompanied by processing of the pre(pro)protein into one of the mature forms of the protein.

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Allelic form. One of the two or more naturally occurring alternative forms of a gene product.

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Chromosome. Thread-like structures into which the hereditary material of cells is associated.

Genome. The total genetic information of cells or-



ganised in the chromosomes.

Maturation form. One of two or more naturally occurring forms of a gene product procured by specific processing, e.g. specific proteolysis.

5 By maturation forms of preprothaumatin are meant prothaumatin, prethaumatin (= preprothaumatin without a carboxylterminal sequence of 6 amino acids) and thaumatin (EP-PA 54330 and EP-PA 54331).
10 By maturation forms of preprochymosin are meant prochymosin, pseudo-chymosin and chymosin (EP-PA 77109).

Plus strand. DNA strand whose nucleotide sequence
15 is identical with the mRNA sequence, with the proviso that uridine is replaced by thymidine.

The microbial cloning vehicles - containing (a) the various forms of the regulons and terminators (indicated with the suffix -01, -02, etc. in the plasmids
20 described in the specification) of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) operon of S. cerevisiae, (b) structural genes encoding preprothaumatin and preprochymosin and their various
25 maturation forms, (c) various hybrid forms of said structural genes encoding maturation forms of preprothaumatin or preprochymosin with special signal sequences and (d) various chemically synthesized DNA-sequences - are produced by a number of steps, the most
30 essential of which are:

1. Isolation of clones containing the GAPDH operon of S. cerevisiae.
2. Isolation of the GAPDH promoter/regulation region
35 and its introduction into plasmids encoding thaumatin-precursors.
3. Introduction of the GAPDH promoter/regulation



- region into plasmids encoding chymosin-precursors.
4. Reconstitution of the original GAPDH promotor/
regulation region in plasmids encoding prepro-
thaumatin by introduction of a synthetic DNA frag-
ment (Fig. 7A, Fig. 8).
 5. Reconstitution of the original GAPDH promoter/
regulation region in plasmids encoding
(pre)(pro)(pseudo)chymosin by introduction of a
synthetic DNA fragment.
 6. DNA-synthesis.
 7. Structural features of the GAPDH promoter/regula-
tion region.
 8. Insertion of fragments of the GAPDH transcription
termination/polyadenylation region in combination
with the central transcription termination signal
of phage M13RF downstream of genes encoding
pseudochymosin.
 9. Introduction of the 2 micron DNA replication origin
and the yeast leu 2 gene in plasmids encoding
thaumatin-precursors and chymosin-precursors.
 10. The introduction of GAPDH transcription termination/
polyadenylation regions into pURY plasmids.
 11. Construction of an E. coli-yeast shuttle vector
widely applicable for gene-expression in yeast.
 12. Expression in K. lactis of the preprothaumatin
encoding gene under control of the promoter/
regulation region of the GAPDH encoding gene of
S. cerevisiae.
 13. Integration of structural genes under control of
the GAPDH promoter/regulation region into the yeast
chromosome.
 14. Chemical synthesis of structural genes and con-
struction of synthetically chimeric genes.
- The following detailed description will illustrate the
invention.
1. Isolation of clones containing the glyceraldehyde-



3-phosphate dehydrogenase (GAPDH) operon of S. cerevisiae.

5 A DNA pool of the yeast S. cerevisiae was prepared
in the hybrid E. coli-yeast plasmid pF1 1 [M.
Chevallier et al. Gene 11, 11-10 (1980)] by a method
similar to the one described by M. Carlson and D.
Botstein, Cell 28, 145-154 (1982). Purified yeast
10 DNA was partially digested with restriction
endonuclease Sau 3A and the resulting DNA fragments
(with an average length of 5 kb) were ligated by T4
DNA ligase in the dephosphorylated Bam HI site of
pF1 1. After transformation of CaCl₂-treated E.
coli cells with the ligated material a pool of about
15 30.000 ampicillin resistant clones was obtained.
These clones were screened by a colony hybridization
procedure [R.E. Thayer, Anal. Biochem., 98, 60-63
(1979)] with a chemically synthesized and ³²P-
labelled oligomer with the sequence
20 5'TACCAGGAGACCAACTT3'.
According to data published by J.P. Holland and M.J.
Holland [J. Biol. Chem., 255, 2596-2605, (1980)]
this oligomer is complementary with the DNA sequence
encoding aminoacids 306-310 (the wobble base of the
25 last amino acid was omitted from the oligomer) of
the GAPDH gene. Using hybridization conditions
described by R.B. Wallace et al., Nucleic Acid Res.
9, 879-894 (1981), six positive transformants could
be identified. One of these harboured plasmid pF1 1-
30 33. The latter plasmid contained the GAPDH gene
including its promoter/regulation region and its
transcription termination/polyadenylation region.

35 The approximately 9 kb long insert of pF1 1-33 has
been characterized by restriction enzyme analysis
(Fig. 1) and partial nucleotide sequence analysis
(Figs. 2 and 3).



Note: Unless stated otherwise, all enzyme incubations were carried out under conditions described by the supplier. Enzymes were obtained from Amersham, Boehringer, BRL or Biolabs.

2. Isolation of the GAPDH promoter/regulation region and its introduction into plasmids encoding thaumatin-precursors (Fig. 4).

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On the basis of the restriction enzyme analysis and the nucleotide sequence data of the insert of plasmid pFl 1-33, the RNA initiation regulation region of the GAPDH gene was isolated as an 800 nucleotides long Dde I fragment. To identify this promoter fragment, plasmid pFl 1-33 was digested with Sal I and the three resulting DNA fragments were subjected to a Southern hybridization test with the chemically synthesized oligomer [E.M. Southern, J.Mol.Biol. 98, 503-517 (1975)]. A positively hybridizing 4.3 kb long restriction fragment was isolated on a preparative scale by electroelution from a 0.7% agarose gel and was then cleaved with Dde I. Of the resulting Dde I fragments only the largest one had a recognition site for Pvu II, a cleavage site located within the GAPDH promoter region (Fig. 1). The largest Dde I fragment was isolated and incubated with Klenow DNA polymerase and four dNTP's (A.R. Davis et al., Gene 10, 205-218 (1980)) to generate a blunt-ended DNA molecule.

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After extraction of the reaction mixture with phenol/chloroform (50/50 v/v), passage of the aqueous layer through a Sephadex G50 column and ethanol precipitation of the material present in the void volume, the DNA fragment was equipped with the ³²P-labelled Eco RI linker 5'GGAATTC3' by



incubation with T4 DNA ligase. Owing to the Klenow DNA polymerase reaction and the subsequent ligation of the Eco RI linker, the original Dde I sites were reconstructed at the ends of the promoter fragment.

5 To inactivate the ligase the reaction mixture was heated to 65°C for 10 minutes, then sodium chloride was added (final concentration 50 mmol/l) and the whole mix was incubated with Eco RI. Incubation was terminated by extraction with phenol/chloroform,

10 the DNA was precipitated twice with ethanol, re-suspended and then ligated into a suitable vector molecule. Since the Dde I promoter fragment was equipped with Eco RI sites it can be easily introduced into the Eco RI site of pUR 528, pUR 523 and pUR 522 (EP-PA 54330 and EP-PA 54331) to create

15 plasmids in which the yeast promoter is adjacent to the structural genes encoding thaumatin precursors. The latter plasmids were obtained by cleavage of pUR 528, pUR 523 and pUR 522 with Eco RI, treatment

20 of the linearized plasmid molecules with (calf intestinal) alkaline phosphatase to prevent self-ligation and incubation of each of these vector molecules, as well as the purified Dde I promoter fragment, with T4 DNA ligase. Transformation of the

25 various ligation mixes in CaCl₂-treated E. coli HB101 cells yielded several ampicillin resistant colonies. From some of these colonies plasmid DNA was isolated [H.C. Birnboim and J. Doly, Nucleic Acids Res. 7, 1513-1523 (1979)] and incubated with

30 Pvu II to test the orientation of the insert.

In the nomenclature plasmids containing the Eco RI (Dde I) GAPDH promoter fragment in the correct orientation (i.e. transcription from the GAPDH

35 promoter occurs in the direction of a downstream located structural gene) are indicated by the addendum-01 to the original code of the plasmid



(for example pUR 528 is changed into pUR 528-01; see Fig. 4).

5 To facilitate manipulation of plasmids containing the Eco RI promoter fragment, one of the two Eco RI sites was destroyed. Two μ g of plasmid DNA (e.g. pUR 528-01) was partially digested with Eco RI and then incubated with 5 units Mung bean nuclease (obtained from P.L. Biochemicals Inc.) in a total
10 volume of 200 μ l in the presence of 0.05 moles per l sodium acetate (pH 5.0), 0.05 moles/l sodium chloride and 0.001 moles/l zinc chloride for 30 minutes at room temperature to remove sticky ends. The nuclease was inactivated by addition of SDS to
15 a final concentration of 0.1% [D. Kowalski *et al.*, *Biochemistry* 15, 4457-4463 (1976)] and the DNA was precipitated by the addition of 2 volumes of ethanol (in this case the addition of 0.1 volume of 3 moles/l sodium acetate was omitted). Linearized
20 DNA molecules were then religated by incubation with T4 DNA ligase and used to transform CaCl_2 -treated *E. coli* cells. Plasmid DNA isolated from ampicillin resistant colonies was tested by cleavage with Eco RI and Mlu I for the presence of
25 a single Eco RI site adjacent to the thaumatin gene (cf Fig. 4).

Plasmids containing the GAPDH promoter fragment, but having only a single Eco RI recognition site
30 adjacent to the ATG initiation codon of a downstream located structural gene, are referred to as -02 type plasmids (for example: pUR 528-01 is changed into pUR 528-02; see Fig. 4).

35 3. Introduction of the GAPDH promoter/regulation region into plasmids encoding chymosin-precursors.



To construct plasmids containing the GAPDH promoter/regulation region adjacent to structural genes encoding chymosin precursors, use was made of plasmid pUR 528-02 (cf 2) digested with Eco RI and Hind III as a vector molecule in which various structural genes were inserted. To overcome the problem that all of the (pre)(pro)(pseudo)chymosin encoding plasmids (pUR 1524, pUR 1523 and pUR 1521, respectively, as described in EP-PA 77109) contain an additional Eco RI site within the structural gene, a Hind III digestion was carried out in combination with a partial Eco RI digest. Restriction fragments containing the intact and isolated gene were extracted from the 1% agarose gel and added to a T4 DNA ligation mix together with the vector. The vector was prepared by digesting pUR 528-02 with Hind III and Eco RI, treating the resulting fragments with phosphatase and isolation of the largest fragment from a 0.7% agarose gel. After transformation of *CaCl₂*-treated *E. coli* cells with the ligation mix and selection for ampicillin resistant colonies, plasmids containing the GAPDH promoter/regulation fragment adjacent to the (pre)(pro)-(pseudo)chymosin encoding structural genes in the appropriate orientation could be isolated. In a similar way, plasmid pUR 1522 can be converted into plasmid pUR 1522-02.

Plasmids containing the GAPDH promoter fragment and the genes coding for prepro-, pro-, pseudochymosin and chymosin are referred to as pUR 1524-02, pUR 1523-02, pUR 1521-02 and pUR 1522-02, respectively.

4. Reconstitution of the original GAPDH promoter/regulation region in plasmids encoding preprothrombin by introduction of a synthetic DNA fragment (Fig. 7A, Fig. 8).



As shown by the nucleotide sequence depicted in Fig. 2, the Eco RI (Dde I) GAPDH promoter fragment contains the nucleotides -844 to -39 of the original GAPDH promoter/regulation region. Not contained in this promoter fragment are the 38 nucleotides preceding the ATG initiation codon of the GAPDH encoding gene. The latter (38) nucleotide fragment contains the PuCACACA sequence, which is found in several yeast genes. Said PuCACACA sequence located about 20 bp upstream of the translation start site [M.J. Dobson et al., Nucleic Acid Res., 10, 2625-2637 (1982)] provides the nucleotide sequence which is optimal for protein initiation [M. Kozak, Nucleic Acids Res. 9, 5233-5252 (1981)]. Moreover, as shown in Fig. 6, these 38 nucleotides allow the formation of a small loop structure which might be involved in the regulation of expression of the GAPDH gene.

On the basis of the above-mentioned arguments, introduction of the 38 nucleotides between the Dde I promoter-fragment and the ATG codon of a downstream located structural gene was considered necessary to improve promoter activity as well as translation initiation.

As outlined in Fig. 7A the missing DNA fragment was obtained by the chemical synthesis of two partially overlapping oligomers. The Sac I site present in the overlapping part of the two oligonucleotides was introduced for two reasons: (i) to enable manipulation of the nucleotide sequence immediately upstream of the ATG codon including the construction of poly A-tailed yeast expression vectors (see 11); (ii) to give a cleavage site for an enzyme generating 3'-protruding ends that can easily and reproducibly be removed by incubation with T4 DNA



polymerase in the presence of the four dNTP's. Equimolar amounts of the two purified oligomers were phosphorylated at their 5'-termini, hybridized [J.J. Rossi et al., (1982), J. Biol. Chem. 257, 9226-9229] and converted into a double-stranded DNA molecule by incubation with Klenow DNA polymerase and the four dNTP's under conditions which have been described for double-stranded DNA synthesis [A.R. Davis et al., Gene 10, 205-218 (1980)]. Analysis of the reaction products by electrophoresis through a 13% acrylamide gel followed by autoradiography showed that more than 80% of the starting single-stranded oligonucleotides were converted into double-stranded material. The DNA was isolated by passage of the reaction mix over a Sephadex G50 column and ethanol precipitation of the material present in the void volume. The DNA was then phosphorylated by incubation with polynucleotide kinase and digested with Dde I. To remove the nucleotides cleaved off in the latter reaction, the reaction mix was subjected to two precipitations with ethanol.

As shown in Fig. 8, cloning of the resulting synthetic DNA fragment was carried out by the simultaneous ligation of this fragment and a Bgl II-Dde I GAPDH promoter regulation fragment in a vector molecule from which the Eco RI site preceding the ATG initiation codon was removed by Mung bean nuclease digestion (cf. 2). The Bgl II-Dde I promoter/regulation fragment was obtained by digestion of plasmid pUR 528-02 with Dde I and Bgl II. Separation of the resulting restriction fragments by electrophoresis through a 2% agarose gel and subsequent isolation of the fragment from the gel yielded the purified 793 nucleotides long promoter/regulation fragment. In the plasmid pUR



528-02 the nucleotide sequence preceding the ATG codon is 5'-GAATTCATG- 3' (EP-PA 54330 and EP-PA 54331), which is different from the favourable nucleotide sequence given by M. Kozak [Nucleic Acids Res. 9, 5233-5252 (1981)]. Since our aim was to reconstitute the original GAPDH promoter/regulation/protein initiation region as accurately as possible, the Eco RI site was removed in order to ligate the synthetic DNA fragment to the resulting blunt-end. Removal of the Eco RI site was accomplished by Mung bean nuclease digestion of Eco RI-cleaved pUR528-02 DNA (see 2).

Subsequently the plasmid DNA was digested with Bgl II and incubated with phosphatase. After separation of the two DNA fragments by electrophoresis through a 0.7% agarose gel, the largest fragment was isolated and used as the vector in which the Bgl II-Dde I promoter fragment as well as the -Dde I-treated- synthetic DNA fragment were ligated. Plasmids in which the Dde I promoter/regulation fragment together with the Sac I recognition site containing the synthetic DNA fragment are introduced are indicated by the addendum -03 (for example: pUR 528-02 is changed into pUR 528-03).

Similar results can be obtained with plasmids containing one of the maturation forms of preprothaumatin as the structural gene, i.e. prethaumatin, prothaumatin and thaumatin, which will result in plasmids pUR 522-03, pUR 523-03 and pUR 520-03, respectively.

In order to reconstitute the original GAPDH promoter/regulation region as accurately as possible, the Sac I site was removed from plasmid pUR 528-03. (Fig. 9). This was accomplished by



digestion of the plasmid DNA with Sac I to generate a linearized plasmid molecule with protruding 3' ends. These ends were then made blunt-ended using the 3'-exonuclease activity of T4 DNA polymerase in the presence of the four dNTP's [T. Maniatis et al. in Molecular Cloning; Cold Spring Harbor Laboratory 117-120 (1982)]. Circularisation of the linear plasmid was accomplished by using T4 DNA ligase.

Plasmids from which the Sac I site present in the synthetic DNA fragment is removed are referred to as -04 type plasmids (for example: pUR 528-03 is changed into pUR 528-04; see Fig. 9).

Similar results can be obtained with plasmids containing a structural gene for one of the maturation forms of preprothaumatin, i.e. prethaumatin, prothaumatin and thaumatin, which will result in e.g. plasmids pUR 522-04, pUR 523-04 and pUR 520-04, respectively.

5. Reconstitution of the original GAPDH promoter/regulation region in plasmids encoding (pre)(pro)(pseudo)chymosin by introduction of a synthetic DNA fragment (Fig. 7B, Fig. 10).

To construct (pre)(pro)(pseudo)chymosin encoding plasmids containing the -03 type GAPDH promoter/regulation region (see 4), use can be made of plasmid pUR 528-03 digested with Sac I and Hind III as a vector molecule in which the various structural genes were inserted together with a synthetic DNA fragment (Fig. 10). The (pre)(pro)(pseudo)chymosin encoding genes can be isolated from the plasmids pUR 1524, pUR 1523, pUR 1521 and pUR 1522 (cf. EP PA 77109) respectively by incubation with Sal I in combination with a partial Eco RI digestion to overcome cleavage of the additional Eco RI site in



in the chymosin gene (cf 3). The resulting DNA fragments can then be incubated with Mung bean nuclease (cf 2) followed by a digestion with Hind III. Restriction fragments containing the intact and isolated gene can be purified by electrophoresis through a 1% agarose gel and then extracted from the gel. The synthetic DNA fragment to be used in the constructions is depicted in Fig. 7B.

The vector molecule can be prepared by digestion of plasmid pUR 528-03 with Sac I and Hind III followed by a phosphatase treatment of the restriction fragments and isolation of the largest fragment from the 0.7% agarose gel. Vector molecule, synthetic DNA fragment (Sac I-treated) and the DNA fragment containing the (pre)(pro)(pseudo)chymosin encoding nucleotide sequence can be incubated with T4 DNA ligase and transformed in CaCl₂-treated E. coli cells. Plasmid DNA obtained from ampicillin-resistant colonies can be tested by incubation with various restriction enzymes.

The nomenclature of the newly created plasmids is similar to the nomenclature of the (pre)(pro)-thaumatin-encoding plasmids (pUR 1524-03, pUR 1523-03, pUR 1521-03 and pUR 1522-03). Removal of the Sac I site has been described also (see 4). Removal of the Sac I sites will result in the plasmids pUR 1524-04, pUR 1523-04, pUR 1521-04 and pUR 1522-04, respectively (see Fig. 10).

6. DNA synthesis.

Desired oligonucleotides were synthesized on a polystyrene support using phosphotriester methodology and a library of dimers. [G.A. van der Marel



et al., Recl.Trav.Chim. Pays-Bas 101, 234-241
(1982)]. Chloromethylated polystyrene (1.34
mmoles/gram) was functionalized with 2-(4-
hydroxylphenyl) ethanol [Su-Sun Wang, J.Am.Chem.
5 Soc. 95, 1325-1333 (1973)] and then coupled with 5'
phosphorylated uridine protected as a mixture of 2'
and 3' acetate and levulinyl groups (analogously to
G.A. van der Marel et al. vide supra). This gave a
support with a levulinyl functionality of 130
10 /umoles/g). The synthesis cycle was carried out
in a 20 ml 2-necked pear shaped flask, one neck of
which carried a sintered glass filter. This allowed
the functionalized polystyrene (60 mg) to remain in
the flask throughout the synthesis which consisted
15 of the following steps.(cf. Fig. 11).

- 1). Removal of the "levulinyl" group with hydrazine
hydrate (0.5 mol/l) in propionic acid/pyridine
(1:3 v/v) for 5 minutes.
- 20 2). Washing with 2 x 2 ml pyridine.
- 3). Addition of a fourfold molar excess of the
required dinucleotide anion (60 /umoles)
protected in the 5' position as levulinyl
ester. This was coevaporated twice with
25 pyridine and reduced to approximately 0.5 ml
before adding MSNT (240 /umoles) [C.B. Rees
et al., Tetrahedron Letters 2727-2730 (1978)].
The mixture was shaken for 60 minutes (except
the first cycle which was lengthened to 90
30 minutes).
- 4). Washing with 2 x 2 ml pyridine.
- 5). Addition of acetic anhydride (0.2 ml) and
dimethyl aminopyridine in pyridine (1.5 ml,
0.05 M) to react with any unreacted hydroxyl
35 group during 5 minutes.
- 6). Washing with 2 x 2 ml pyridine.
This cycle was repeated with the appropriate



dimers until the desired sequence had been prepared. The 2- chlorophenyl phosphate protecting groups were removed with 0.3 mol/l 1,1,4,4-tetramethyl guanidinium 2-
5 pyridinealldoximate (C.B. Reese et al., vide supra) in dry acetonitrile for 24 hours. After filtration and washing of the support the base protecting groups (dA = benzoyl, dC = anisoyl, dG = diphenylacetyl) were cleaved with
10 concentrated aqueous ammonia for 60 hours at 50° which also cleaves the sequence from the uridine attached to the support [R. Crea and T. Horn, Nucleic Acids Res., 8, 2331-2348 (1980)]. Filtration of the aqueous phase from the
15 support and evaporation gave a crude mixture which was given a clean-up by chromatography on a short column (40 cm) of Sephadex G50 with 0.05 M triethylammonium bicarbonate as eluent. Collecting and evaporating the first five
20 fractions containing UV absorbing material gave a concentrate suitable for preparative gel electrophoresis.

25 7. Structural features of the GAPDH promoter/regulation region (Fig. 2, Fig. 6)

TATA or TATAAA sequences are believed to play an important role in positioning RNA initiation sites in eucaryotic promoter structures which are
30 recognized by RNA polymerase II [C. Breathnach and P. Chambon, Annu. Rev. Biochem. 50, 349-384 (1981)]. Usually transcription is initiated 25 to 30 nucleotides 3' to such sequences although for yeast varying distances (up to 70 nucleotides; M.J. Dobson etal., Nucleic Acids Res. 10, 2625-2637
35 (1982)) have been described. According to the nucleotide sequence data obtained during the



present investigations for the GAPDH promoter/regulation region, this structure contains two additional sets of TATA and TATAAA sequences which are located towards the ends of the promoter fragment (Fig. 2). Most likely the clustered 5'TATATAA 3' sequence occurring around position -130 is responsible for transcription of the GAPDH encoding gene. The two other TATA and TATAAA sequences present around positions -608 and -770 are possibly involved in regulation of GAPDH expression (see below; P.K. Maitra and Z. Lobo, J. Biol. Chem., 246, 489-499 (1971)). Besides these RNA initiation signals, the GAPDH promoter/regulation fragment contains various nucleotide sequences which are implicated in transcription termination. K.S. Zaret and F. Sherman [Cell 28, 563-573 (1982)] found the sequences TAG-N-TAGT-N'-TTT or TAG-N"-TATGT-N""-TTT, in which N,N',N" and N"" represent the variable distances between the groups, in the 3' flanking sequences of a majority of yeast genes examined. Identical or similar sequences occur in the GAPDH promoter/regulation fragment around position -625 (TAT-N₄-TAGT-N₅-TTT), around position -324 (TAC-N₁₃-TAGT-N₁₇-TTA, around position -192 (TAC-N₉-TATGT-N₁-TTT) and around position -180 (TAT-N₁₇-TAGT-N₂₃-TTT). In Fig. 6 these postulated termination signals are represented by slashes. The largest open translation reading frame present on the GAPDH promoter/regulation fragment extends from position -450 to -337 and encodes a peptide of 38 amino acids long. Since the TATA box around position -608 precedes the open reading frame it might well be that the putative peptide is translated from a transcript initiated downstream of this TATA sequence. Particularly interesting is the observation that the ATG initiation codon of the peptide forms part of a



secondary structure which is generated upon base pairing of the nucleotides extending from -448 to -436 with the nucleotides extending from -419 to -407. These features are reminiscent of the situation described for the yeast leu 2 gene [A. Andreadis et al., Cell 31, 319-325 (1982)] and together with the presence of the small stem/loop structure preceding the ATG codon of the GAPDH encoding gene they might be involved in regulating the expression of the latter gene.

8. Insertion of fragments of the GAPDH transcription termination/polyadenylation region in combination with the central transcription termination signal of phage M13 RF downstream of genes encoding pseudochymosin (Fig. 12).

To isolate fragments of the GAPDH transcription termination/polydenylation region, plasmid pF1-33 was cleaved with restriction enzyme Afl II. Digestion with this enzyme yielded two fragments, the smaller of which has a length of 1307 nucleotides and encompasses the GAPDH termination/ polyadenylation region from position 11 to 1317 (Fig. 12). The latter fragment was isolated, incubated with Mung bean nuclease to generate blunt ends (cf 2) and then equipped with the Bam HI linker (5'CCGGATCCGG3') using T4 DNA ligase. Owing to the presence of a naturally occurring Bam HI site in the middle (around position 690) of the Afl II fragment isolated, digestion of the ligation mix with Bam HI resulted in two fragments (A and B; Fig. 12). The larger of these two fragments (A) has a length of 677 nucleotides and contains the nucleotide sequence region which is located immediately downstream of the TAA translation termination codon. Attempts to subclone the purified fragment A in the



the Bam HI site of pBR322 proved to be unsuccessful since very few transformants were obtained and these transformants always contained fragment A as well as fragment B in various orientations: (cf. plasmid 294-17 depicted in Fig. 12).

To overcome this instability of fragment A, use was made of the central transcription termination signal of bacteriophage M13 RF [L. Edens et al., Nucleic Acids Res. 2, 1811-1820 (1975)]. M13 RF was digested with Taq I and the resulting fragments were made blunt end by incubation with Mung bean nuclease. The fragments were then equipped with a Hind III linker (5'AGAAGCTTCT3') using T4 DNA ligase followed by a digestion with Hind III. The DNA was precipitated from the reaction mix by the addition of two volumes ethanol. The precipitate was resuspended and the various restriction fragments were separated by electrophoresis through a 4% acryl-amide gel. From this gel the 441 nucleotides long fragment containing the central transcription termination signal was isolated.

After cleavage of the purified fragment with Sau 3A, the fragment containing the nucleotides 1509 to 1717 [P.M.G. van Wezenbeek et al., Gene 11, 129-148 (1980)] was closed in pBR322 which had been digested with Hind III and Bam HI (Fig. 12).

To create plasmids in which the 3'-untranslated region of the genes encoding (pre)(pro)(pseudo) chymosin was replaced by the 3'-untranslated region of the GAPDH encoding gene, plasmid pUR 1521-02 was isolated from an E. coli strain deficient in adenine methylase and cleaved with restriction endonuclease Bcl I. This enzyme recognizes the (unmethylated) sequence 5'TGATCA 3' and cleaves the



(pre)(pro)(pseudo)chymosin encoding genes within their translation termination codon TGA. To generate suitable vector molecules in which both fragment A and the M13 transcription termination signal could be cloned, the Bcl I-cleaved plasmid molecules were incubated first with Sal I, followed by alkaline phosphatase. The larger of the two restriction fragments generated was isolated from the 0.7% agarose gel. For the isolation of fragment A, plasmid 294-17 was digested with Hind III and cleaved partially with Bam HI. The 1020 nucleotides long Hind III-Bam HI fragment containing the Hpa I site was obtained by electro-elution from the 1% agarose gel. The latter fragment, the vector and the 485 nucleotide long Hind III-Sal I fragment obtained from pBR322 containing the M13 transcription termination signal were incubated with T4 DNA ligase and transformed in CaCl₂-treated E. coli cells. This finally yielded the plasmid pUR 1521-12. Similar results can be obtained with plasmids pUR 1524-02, pUR 1523-02 and pUR 1522-02, which will result in plasmids pUR 1524-12, pUR 1523-12 and pUR 1522-12, respectively.

In the nomenclature plasmids containing fragment A and the M13 transcription termination signal downstream of a newly inserted structural gene are indicated by replacing the addendum -Ox with -1x.

9. Introduction of the 2 micron DNA replication origin and the yeast leu 2 gene in plasmids encoding thaumatin-precursors and chymosin-precursors (Fig. 13, Fig. 14).

The E. coli-yeast shuttle vector pMP81 [Fig.13 ; C.P. Hollenberg, Current Topics in Microbiol. and Immunol., 96, 119-144, (1982)] consists of plasmid



pCRI [C. Covey et al., MGG, 145, 155-158 (1976)]
and a double Eco RI fragment of pJDB 219 [J.D.
Beggs, Nature, 275, 104-109 (1978)] carrying both
the leu 2 gene and the yeast 2 micron DNA
5 replication origin. The latter two functions can
be excised from pMP81 by a digestion with Hind III
and Sal I. The resulting 4.4 kb long restriction
fragment was introduced into the various pBR 322
derivatives containing genes encoding thaumatin-
10 precursors in combination with the various forms of
the GAPDH promoter region of S. cerevisiae. A
similar procedure was used to introduce the 4.4 kb
long restriction fragment into the various pBR322
derivatives containing genes encoding chymosin-
15 precursors in combination with the GAPDH promoter
region of S. cerevisiae.

The introduction of the 2 micron replication origin
and leu 2 gene containing Hind III-Sal I fragment
20 into the various plasmids was accomplished by
cleavage of the E. coli plasmids with Hind III and
Sal I (cf. Fig. 14) and a subsequent treatment of
the resulting fragments with phosphatase. After
separation of the fragments by electrophoresis
25 through a 1% agarose gel, the largest fragment was
isolated, mixed with the purified Hind III-Sal I
fragment obtained from pMP81, ligated with T4 DNA
ligase and transformed to CaCl₂-treated E. coli
cells.

30 In the pseudochymosin encoding plasmid containing
termination fragment A (pUR 1521-12; cf. Fig. 12),
the 4.4 kb long Hind III-Sal I fragment was in-
serted as well. For this purpose plasmid pUR
35 1521-12 was digested with both Hind III and Sal I
(to remove the M13 transcription termination
region) after which the 2 micron origin and leu 2



gene containing fragment from pMP81 could be inserted. Unexpectedly, this plasmid construction (pURY 1521-12) proved to be stable in E. coli.

5 From some of the ampicillin-resistant transformants plasmid DNA was isolated and subjected to restriction enzyme analysis. Plasmids containing the correct insert were purified by CsCl-ethidium bromide density gradient centrifugation and used to transform S. cerevisiae AH22 (A. Hinnen et al.,
10 Proc.Natl.Acad.Sci.USA 75, 1929-1933 (1978)) according to the procedure of J.D. Beggs, Nature 275, 104-109 (1978). This resulted in plasmids indicated by the letter code pURY but having the
15 same figure codes (cf. Fig. 14 in which the conversion of plasmid pUR 528-03 into pURY 528-03 is indicated). Similarly, plasmids pUR 522-02, pUR 523-02, pUR 1524-02, pUR 1523-02 and pUR 1521-02 were converted into their corresponding pURY plasmids.
20

With the availability of yeast transformants containing the newly constructed plasmids, the effects of plasmid variation on gene expression could be
25 monitored. For this purpose an enzyme-linked immunosorbent assay [Elisa: A Voller et al., Bull. World Health Organ. 53, 55-65 (1976)] for the thaumatin was developed and used to quantitate the amounts of thaumatin-like protein present in yeast
30 extracts. The results obtained in these experiments show that upon the introduction of the -02 or -03 type GAPDH promoter in pURY 528 (cf. 2, 4), thaumatin synthesis is increased by more than two orders of magnitude (more than 100 times). Upon the
35 introduction of the 280 nucleotides long promoter fragment described by J.P. Holland and M.J. Holland [J.Biol.Chem., 255, 2596-2605, (1980)], however,



thaumatin synthesis increased by order of magnitude of only one (about 10 times), hereby demonstrating the important role played by upstream sequences of the GAPDH promoter in its functioning, which is
5 contrary to the conclusions of Holland and Holland [cf. J.Biol.Chem. 254, 9839-45 (1979)].

In another experiment the expression of preprothaumatin and prothaumatin encoding genes was compared. As expression of the prothaumatin encoding
10 gene turned out to be almost negligible, this result clearly demonstrated the enormous impact which signal sequences can have on gene expression. The importance of the processing step was further
15 substantiated by the results shown in Fig. 20. The latter experiment demonstrates that yeast cells harbouring plasmids encoding preprothaumatin are able to produce a thaumatin-like protein with a molecular weight which is practically the same as
20 the molecular weight of thaumatin II molecules isolated from the plant.

This suggests that yeast is able to correctly process the plant signal sequence. Recently obtained
25 data on the amino-terminal amino acid sequence of yeast-synthesized thaumatin have provided definite evidence for this notion.

In conclusion, the results obtained strongly suggest that the signal sequence plays an important
30 role in either stimulating protein synthesis or increasing protein stability in yeasts. It is not yet sure whether prothaumatin or thaumatin is produced by yeasts in view of the smaller difference in
35 molecular weight (about 3%) between these proteins, whereas the difference with preprothaumatin (about 10%) is easily detectable as was demonstrated in



Fig. 20.

10. The introduction of GAPDH transcription termination/polyadenylation regions into pURY plasmids.

5 One of the possibilities to introduce GAPDH transcription termination/polyadenylation regions into pURY plasmids is to use the unique Hind III restriction site of these plasmids for insertion of various parts of the Afl II fragment depicted in Fig. 12. For example, the insertion of transcription termination fragment A (cf. Fig. 12) can be carried out as follows. Cleavage of plasmid pURY 528-03 with Hind III and a subsequent Mung bean
10 nuclease digestion (cf. 2) will yield a linearized and blunt-ended plasmid molecule (cf. Fig 14). Incubation of this DNA with T4 DNA ligase and a suitable Bam HI linker will equip the fragment with Bam HI sites (cf. 8). Upon transformation of the Bam HI-cleaved and religated product into E. coli, pURY 528-03 plasmids in which the Hind III site is replaced by a Bam HI site can be recovered. Into this newly created Bam HI site transcription termination
20 fragment A can be inserted. Digestion of the latter construction with Hpa I will indicate whether or not fragment A is inserted at the correct orientation, since in the 2 micron DNA sequence an additional Hpa I site is available.

30 Using the same approach but different linker molecules, different terminator fragments can be introduced at various sites in the plasmid molecule.

11. Construction of an E. coli-yeast shuttle vector widely applicable for the expression of foreign
35 genes in yeast (Fig. 15).

Derivatives of the E. coli-yeast shuttle vectors



described under 5 and 9, useful as generally applicable yeast expression vectors, can be prepared. In these derivatives the GAPDH promoter/regulation region including a chemically synthesized DNA fragment can be used for DNA initiation, whereas transcription termination can be effected by the transcription termination/polyadenylation region of the "Able" operon of the 2 micron DNA. Insertion of foreign genes in these expression vectors can be accomplished by homopolymer (poly dA) tailing of the Sac I site in the promoter and the Hind III site present in the "Able" operon in combination with poly dT tailing of the nucleotide sequence to be inserted.

For the preparation of the vector molecule, plasmid pURY 528-03 can be cleaved with Hind III and then incubated with Klenow DNA polymerase and the four dNTP's to generate a blunt-ended, linearized DNA molecule (Fig. 15). Subsequently this DNA molecule can be cleaved with Sac I and of the two resulting DNA fragments the larger can be isolated from a 0.7% agarose gel and incubated with terminal transferase and dATP under conditions described by G. Deng and R. Wu [Nucleic Acids Res. 9, 4173-4188 (1981)]. The time of incubation has to be such that the tail added to the 3' end generated by cleavage with Sac I has a length of about 20 dATP residues.

The introduction of a foreign gene into the poly dA-tailed expression vector can be carried out by incubating the DNA containing this gene with a set of restriction enzymes such that the desired gene can be cleaved as close as possible upstream of the translation initiation codon and downstream of the translation termination codon of this gene. Since promoter regions can be preceded by transcription



termination signals, it is important that the original promoter is not contained within the resulting DNA fragment. After purification, the trimmed DNA fragment can be equipped with poly dT tails.

5

If the gene to be inserted is obtained by reverse transcription of an mRNA molecule, the poly dT tails can be directly added to the S1 nuclease-treated double-stranded DNA molecule. In all cases the time of incubation with terminal transferase must be chosen such that poly dT tails with a length of about 20 nucleotides are generated. The DNA to be inserted and the poly dA-tailed vector molecule can then be hybridized by incubation at 65°C for 10 minutes, followed by cooling down the hybridization mixture slowly to room temperature. The mixture can be subsequently transformed into CaCl₂-treated E. coli cells. Plasmid DNA isolated from ampicillin-resistant transformants can then be used to transform yeast cells.

12. Expression in K. lactis of the preprothaumatin encoding gene under control of the promoter/regulation region of the glyceraldehyde-3-phosphate dehydrogenase encoding gene of S. cerevisiae (Fig. 16, Fig. 17).

The E. coli-yeast shuttle vector pEK 2-7 consists of plasmid YRp7 [D.T. Stinchcomb et al., Nature 282, 39-43 (1979)] containing the 1.2 kb KARS-2 fragment. Owing to the presence of the yeast trp 1 gene, plasmid pEK 2-7 can be maintained in K. lactis SD11 (lac 4, trp 1; cf. pages 18 and 19 of European Patent Application N° 0 096 910; A1).

35

To demonstrate the functionality of the promoter/regulation region of the GAPDH encoding gene in



5 K. lactis, plasmid pUR 528-03 (Fig. 8) has been equipped with both KARS-2 and the trp 1 gene. The latter two functions were excised from pEK 2-7 by a digestion with Bgl II followed by the isolation
10 from a 0.7% agarose gel of the smallest fragment generated. This purified fragment was then inserted in the dephosphorylated Bgl II cleavage site of pUR 528-03 by incubation with T4 DNA ligase. Trans-formation of the ligation mix in CaCl₂-treated E. coli
15 cells yielded plasmid pURK 528-03 (Fig. 16). Transformants generated by the introduction of the latter plasmid into K. lactis SD 11 cells by the procedure described in European Patent Application N° 0 096 910; A1 could be shown to synthesize pre-prothaumatin (Fig. 17).

By techniques similar to those mentioned above, plasmid pURY 528-03 was also equipped with KARS-2 and the yeast trp 1 gene and introduced into K. lactis
20 SD 11 (Fig. 16). Using the same detection procedure, K. lactis cells carrying pURK 528-33 could also be shown to synthesize preprothaumatin (Fig. 17). Preprothaumatin production in cells containing pURK 528-33 was, however, slightly
25 higher than in cells containing pURK 528-03. Since similar observations have been made by C. Gerbaud et al. [Gene 5, 233;253 (1979)] in the expression of the yeast ura 3 gene upon insertion of this gene within the coding region "Able" of the 2 micron DNA,
30 it is very likely that the enhanced expression of preprothaumatin by pURK 528-33 is due to efficient transcription termination events in the transcription termination/polyadenylation region of the "Able" operon. This observation indicates that the
35 presence of an efficient transcription termination/polyadenylation region downstream of a structural gene transcribed by the GAPDH promoter/regulation



region is an important factor in optimizing gene expression.

- 5 13. Integration of structural genes under the control of the GAPDH promoter/regulation region into the yeast chromosome (Fig. 18; Fig. 19).

10 Integration of DNA sequences encoding either a heterologous or homologous structural gene under transcriptional control of the GAPDH promoter/regulation region and, optionally, the GAPDH termination/polyadenylation region into the yeast genome, can be achieved on the basis of techniques described by R.J. Rothstein [in Methods in Enzymology 101, 202-211 (1983)] and K. Struhl [Nature 15 305, 391-397 (1983)]. The criteria to apply these techniques are the availability of (i) suitable marker genes, (ii) cloned DNA sequences homologous with DNA sequences present on the yeast genome and 20 (iii) the availability of an intact, homologous or heterologous, protein-encoding DNA sequence.

25 Having the marker genes (e.g. leu 2, trp 1, his 3) and the protein-encoding DNA sequence (e.g. the sequences encoding thaumatin-precursors or chymosin-precursors) available, the latter sequences can be integrated into the yeast genome by homologous recombination events either between GAPDH promoter and terminator sequences (cf. Fig. 30 19) or between the marker genes. In the latter approach, which offers a variety of integration sites, the foreign protein encoding DNA sequence is integrated within the "wild type" marker gene, hereby destroying the function of this gene.

35

14. Chemical synthesis of structural gene.

Construction of synthetically chimeric genes.



Expression experiments of the various structural genes of preprothaumatin and preprochymosin - located on the 2/um DNA vector and under control of the GAPDH promoter/regulation region and the GAPDH termination region - in S. cerevisiae resulted in an expression yield that was considered not yet economically attractive for the fermentative production of these proteins. The expression of preprothaumatin and preprochymosin genes located on the KARS-vector did not match economic feasibility either.

Another problem observed during expression experiments was that preprothaumatin was processed correctly in both S. cerevisiae and K. lactis into thaumatin (Fig. 20) (it is not clear whether the 6 amino acids on the C-terminus are also removed during processing); however, such a correct processing could not be detected with preprochymosin.

Therefore it is advocated to make preprochymosin and preprothaumatin in the preferred codons of S. cerevisiae [J.P. Holland and M.J. Holland; J. Biol.Chem. 255, 2596-2605 (1980)] to increase the expression-yield. The syntheses of these genes can be carried out according to the methods described under 4 and 6. The nucleotide sequences of chymosin and thaumatin in a preferred codon usage are given in Fig. 21 and Fig. 22.

As described under 4 and 6, the synthesis can be carried out by making small (up to 30 nucleotides) single strand DNA fragments with partial overlapping sequences. This method makes it possible that also the various maturation forms of both genes are obtained simultaneously (cf. Fig. 21-22). Moreover, the approach adopted is suitable for



making changes in parts of the sequences. One can use this possibility to replace the leader sequence of preprochymosin (nucleotides -174 to -127, Fig. 21) by various other leader sequences. Two
5 typical yeast leader sequences are that of acid phosphatase [K. Arima et al., Nucleic Acids Res. 11, 1657-1672 (1983)] and that of invertase [R. Taussig and M. Carlson, Nucleic Acids Res. 11, 1943-1954 (1983)]. Examples of nucleotide sequences
10 encoding both leader sequences with codons preferred by yeasts are given in Fig. 23, whereas Fig. 24 gives schematically two designed chimeric acid phosphatase/prochymosin and invertase/prochymosin genes. Because preprothaumatin was processed
15 correctly by the yeast cells, we also designed a chimeric gene of prochymosin and the leader sequence of the preprothaumatin gene (Fig. 24). Based on the physico-chemical considerations about the nature of the yeast leader sequences and the
20 interaction of these leader sequences with the signal recognition protein [P. Walter and G. Blobel, Proc. Natl. Acad. Sci. USA, 77, 7112-7116 (1980)], we also designed two consensus leader sequences (Fig. 25) which can be used to make
25 chimeric genes of these consensus sequences with the prochymosin gene (Fig. 24).



Legends to the figures

- Fig. 1 Restriction endonuclease cleavage map of a region of plasmid pFl 1-33 containing a yeast glyceraldehyde-3-phosphate dehydrogenase operon.
- Fig. 2 Nucleotide sequence of the promoter/regulation region of a glyceraldehyde-3-phosphate dehydrogenase operon cloned in pFl 1-33. TATA and TATAAA sequences are indicated by solid underlining. Presumptive transcription termination signals are underlined with dots. Nucleotide sequences between brackets indicate inverted repeats. The nucleotide sequence encoding the 38 amino acids long peptide is enclosed in a box.
- Fig. 3 Nucleotide sequence of the transcription termination/polyadenylation region of a glyceraldehyde-3-phosphate dehydrogenase operon cloned in pFl 1-33. AATAA sequences are indicated by solid underlining. Presumptive transcription termination signals are underlined with dots.
- Fig. 4 Schematic representation of the insertion of the Eco RI (Dde I) GAPDH promoter/regulation fragment in the preprothaumatin encoding plasmid and removal of an Eco RI cleavage site from the resulting plasmid.
- Fig. 5 Schematic representation of the construction of (pre)(pro)-(pseudo)chymosin encoding plasmids containing the Eco RI (Dde I) GAPDH promoter/regulation fragment.



Fig. 6 Schematic representation of the structure of the GAPDH promoter/regulation region including potential stem and loop structures. Presumptive transcription termination signals are indicated by slashes. The position of the coding sequence for the 38 amino acids long peptide on the fragment is shown by ATG and TAA codons.

Fig. 7a Representation of the various steps involved in the preparation of the synthetic DNA fragment used to reconstitute the original GAPDH promoter/regulation region upstream of preprothaumatin encoding nucleotide sequences.

Fig. 7b Representation of the synthetic DNA fragment used to reconstitute the original GAPDH promoter/regulation region upstream of (pre)(pro)(pseudo)chymosin encoding nucleotide sequences.

Fig. 8 Schematic representation of the introduction of the synthetic DNA fragment in preprothaumatin encoding plasmids.

Fig. 9 Schematic representation of the removal of the Sac I site from the reconstituted GAPDH promoter/regulation region.

Fig. 10 Schematic representation of the introduction of the synthetic DNA fragment in (pre)(pro)(pseudo)chymosin encoding plasmids.

Fig. 11 General scheme for synthesis of DNA fragments on a polystyrene support.



Fig. 12 Schematic representation of an Afl II-Bam HI transcription termination/ polyadenylation fragment obtained from pF1 1-33 and its insertion in combination with the M13 central transcription termination signal, downstream of the nucleotide sequence encoding pseudo-chymosin

Fig. 13 Schematic representation of plasmid pMP81.

Fig. 14 Schematic representation of the introduction of the 2 micron DNA origin of replication and the leu 2 gene obtained from pMP81 by digestion with Hind III and Sal I into preprothaumatin encoding plasmids.

Fig. 15 Schematic representation of an E. coli-yeast shuttle vector widely applicable for the expression of foreign genes in yeast.

Fig. 16 Schematic representation of the introduction of the KARS-2 and trp 1 gene obtained from pEK 2-7 by digestion with Bgl II into prepro-thaumatin encoding plasmids.

Fig. 17 Fluorogram of (^{35}S) labelled thaumatin-like proteins synthesized by K. lactis SD11 cells containing plasmid pEK 2-7 (lane a), plasmid pURK 528-03 (lane b) and plasmid pURK 528-33 (lane c) Yeast transformants were grown for 3 hours on a minimal medium containing ^{35}S -cysteine. Cells were collected by centrifugation, resuspended in 1 ml 2.0 mol/l sorbitol, 0.025 mol/l NaPO_4 pH 7.5, 1 mmol/l EDTA, 1 mmol/l MgCl_2 , 2.5% β -mercaptoethanol, 1 mg/ml zymolyase 60.000 and incubated for 30 minutes at 30°C . Spheroplasts were then centrifuged and lysed by the addition of $270\mu\text{l}$ H_2O , $4\mu\text{l}$ 100 mmol/l PMSF, $8\mu\text{l}$ 250 mmol/l EDTA, $40\mu\text{l}$ 9% NaCl and $80\mu\text{l}$ 5x PBSTDs (50 mmol/l NaPO_4 pH 7.2, 5% Triton X 100, 2.5% deoxycholate, 2.5 % SDS). Immunoprecipitation of thaumatin-like proteins and analysis of precipitated proteins was carried out as described by L. Edens et al, Gene 18, 1-12 (1982).



Fig. 18 Schematic representation of plasmid pEK 2-7.

Fig. 19 Schematic representation of a plasmid which can be used for the insertion of a pseudochymosin-encoding nucleotide sequence under transcriptional control of the GAPDH promoter/regulation and the GAPDH termination/polyadenylation region into the yeast chromosome.

Fig. 20 Fluorogram of (^{35}S) cysteine labelled thaumatin-like protein synthesized by *S.cerevisiae* AH 22 cells containing plasmid pURY 528-03 (lane c) or synthesized in vitro in a wheat germ protein synthesizing system under the direction of mRNA purified from arils of *Thaumatococcus* fruits (lane b). Lane a shows radioactive marker proteins (obtained from Amersham) and lane d shows the position of thaumatin II isolated from the arils of *Thaumatococcus* fruits. Lysis of yeast cells was carried out as described in the legend of fig. 17. Wheat germ translation of mRNA and immunoprecipitation procedures were carried out as described by L. Edens et al, Gene 18, 1-12 (1982).

Fig. 21 Nucleotide sequence of the gene encoding (pre)(pro)(pseudo) chymosin in codon usage preferred by *S. cerevisiae*.

Fig. 22 Nucleotide sequence of the gene encoding (pre)(pro)thamatin in codon usage preferred by *S. cerevisiae*.

Fig. 23 Nucleotide sequence of the genes encoding the leader sequences acid phosphatase and invertase in codon usage preferred by *S. cerevisiae*.

Fig. 24 Schematic representation of the construction of the gene encoding prochymosin provided with the leader sequences of the invertase, acid phosphatase or thaumatin encoding genes, or with the two designed consensus leader sequences.

Fig. 25 Nucleotide sequences of the two designed consensus leader sequences.



CLAIMS

1. A DNA sequence capable of initiating transcription by yeast RNA polymerase II which includes at least part of the regulon region of one of the GAPDH genes of S. cerevisiae, characterized in that it comprises a DNA sequence essentially as given in Fig. 2, and wherein the regulon region is optionally modified to include at least one restriction enzyme cleavage site, to facilitate manipulation of the nucleotide sequence region for protein synthesis initiation.
2. A DNA sequence capable of both termination of the transcription by yeast RNA polymerase II and effecting polyadenylation of the mRNA, which includes at least part of the termination/polyadenylation region belonging to one of the GAPDH genes of S. cerevisiae, characterised in that it comprises a DNA sequence essentially as given in Fig. 3.
3. DNA sequence, which can be inserted into a recombinant DNA plasmid or into a yeast chromosome, comprising:
- (a) a DNA sequence according to claim 1, and
 - (b) one or more structural genes different from the GAPDH genes of S. cerevisiae, and at least two of features (c)-(f),
 - (c) one or more specific DNA sequences capable of terminating the transcription by yeast RNA polymerase II and effecting polyadenylation of the mRNA, and/or
 - (d) one or more selection markers, and/or
 - (e) either one or more nucleotide sequences allowing a stable insertion in a chromosome of yeasts or one or more DNA sequences which regulate DNA replication in yeasts belonging to the genus Saccharomyces or to the genus Kluyveromyces, and/or



- (f) a DNA sequence encoding a signal polypeptide of not more than 30 amino acid residues assisting the translocation of proteins, which DNA sequence is situated upstream of and in the same reading frame as the structural gene.

4. DNA sequence according to claim 3, characterized in that the structural gene of (b) encodes thaumatin or chymosin, or their various allelic or modified forms, which modified forms do not impair the sweet-tasting properties of thaumatin or the milk-clotting properties of chymosin, respectively, or precursors of these thaumatin-like or chymosin-like proteins.

5. DNA sequence according to claim 3, characterized in that the specific DNA sequence of (c) is a DNA sequence essentially as given in Fig. 3.

6. DNA sequence according to claim 3, characterized in that the DNA sequence of (f) encoding a signal polypeptide is selected from the group consisting of DNA sequences encoding

- (a) the signal polypeptide translocating S. cerevisiae invertase, namely Met.Leu.Leu.Gln.Ala.Phe.Leu.Phe.-
Leu.Leu.Ala.Gly.Phe.Ala.Ala.Lys.Ile.Ser.Ala;
- (b) the signal polypeptide translocating S. cerevisiae acid phosphatase, namely Met.Phe.Lys.Ser.Val.Val.-
Tyr.Ser.Ile.Leu.Ala.Ala.Ser.Leu.Ala.Asn.Ala;
- (c) the signal polypeptide of unmaturred forms, of thaumatin-like proteins, namely Met.Ala.Ala.Thr.-
Thr.Cys.Phe.Phe.Phe.Leu.Phe.Pro.Phe.Leu.Leu.Leu.-
Leu.Thr.Leu.Ser.Arg.Ala;
- (d) the signal polypeptide of unmaturred forms of chymosin-like proteins, namely Met.Arg.Cys.Leu.Val.-
Val.Leu.Leu.Ala.Val.Phe.Ala.Leu.Ser.Gln.Gly; and
- (e) two consensus signal polypeptides, namely
Met.Ser.Lys.Ala.Ala.Leu.Ala.Phe.Ile.Ala.Phe.Val.-



Ile.Val.Leu.Ile.Val.Asn.Ala and
 Met.Ser.Lys.Phe.Val.Ile.Val.Leu.Ile.Val.Ala.Ala.-
 Leu.Ala.Phe.Ile.Ala.Asn.Ala.

5 7. DNA sequence according to claim 3, characterized in that either the codons of the structural gene of (b) or the codons of the signal polypeptide-encoding DNA sequence of (f), or both, are modified into codons preferred by yeasts.

10

8. DNA sequence according to claim 7, characterized in that as codons preferred by yeasts the following codons are used:

	GCC or GCT	alanine	TTG	leucine
15	AGA	arginine	AAG	lysine
	AAC	asparagine	ATG	methionine
	GAC or GAT	aspartic acid	TTC	phenylalanine
	TGT	cysteine	CCA	proline
	CAA	glutamine	TCC or TCT	serine
20	GAA	glutamic acid	ACC or ACT	threonine
	GGT	glycine	TGG	tryptophan
	CAC	histidine	TAC	tyrosine
	ATC or ATT	isoleucine	GTC or GTT	valine.

25 9. Process for preparing yeasts containing rDNA sequences, characterized in that DNA sequences as claimed in claim 3 are introduced into Saccharomyces, Kluyveromyces, Debaryomyces, Hansenula, Candida, Torulopsis or Rhodotorula yeasts, either in the form of
 30 plasmids or by incorporation in the yeast genome.

10. Yeast containing an rDNA sequence as claimed in claim 3, either in the form of a plasmid or incorporated in the yeast genome.



11. Process for preparing a protein by cultivation of a yeast containing rDNA sequences, characterized in that a yeast as claimed in claim 10 is used to produce a protein, whereby the rDNA sequence contains a structural gene encoding that protein or a precursor thereof which precursor during processing can form the relevant protein.
12. Protein, produced by a process according to claim 11.



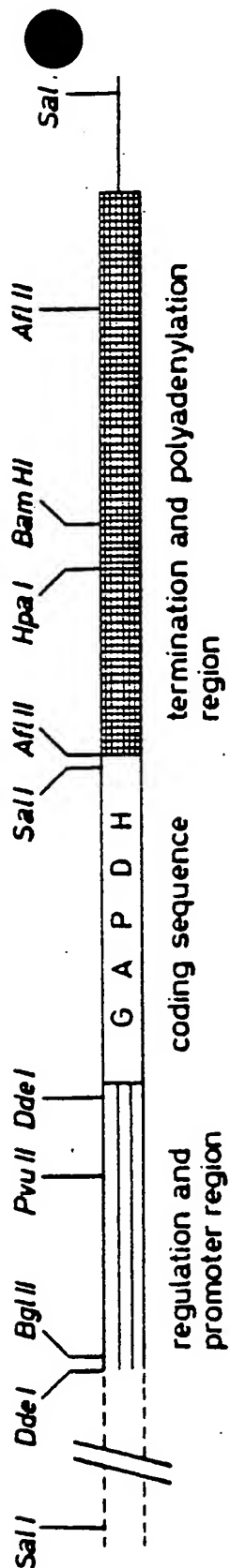


Fig.1.

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-850      -840      -830      -820      -810      -800
GAATTCCTCA GTTTCAGAT CTTTAAATGT CCAAAACCAT TTGAGCCGAT CTAATACTT
-790      -780      -770      -760      -750      -740
CTGTGTTTC ATTAATTTAT AAATTGTACT CTTTAAAGAC ATGGAAGTA CCAACATCGG
-730      -720      -710      -700      -690      -680
TTGAAACAGT TTTTCATTTA CATATGCTTT ATTGGTTTTT CCAGTGAATG ATTATTGTC
-670      -660      -650      -640      -630      -620
GTTACCCCTT CGTAAACTT CAAACACGTT TTTAAGTATT GTTAAAGTTGC TCTTTCGACA
-610      -600      -590      -580      -570      -560
TATATGATTA TCCCTGCGG GCTAAAGTTA AAGATGCAAA AAACAGAAGA CAACCTGAAGT
-550      -540      -530      -520      -510      -500
TAATTTACGT CAATTAAGTT TTCCAGGGTA ATGATGTTTT GGGCTTCCAC TAATTCAATA
-490      -480      -470      -460      -450      -440
AGTATGTCTA GAAATACGTT GTGAAGAGCA TCCAGAAATA ATGAAAAGAA ACAACGAAAC
-430      -420      -410      -400      -390      -380
TGGGTGGCC TGTGTTTCT TTTCTTTACC ACGTGATCTG CGGCATTAC AGGAAGTCGC
-370      -360      -350      -340      -330      -320
GCGTTTTCG CAGTTGTTC AACGCAGCTA CCGCTAAACAA AGCCTAGTGG AACTCGACTG
-310      -300      -290      -280      -270      -260
ATGTGTIAGG GCCTAAACT GGTGGTGACA GCTGAAGTGA ACTATTCAAT CCAATCATGT
-250      -240      -230      -220      -210      -200
CATGGCTGTC ACAAGACCT TGGGGACCGC ACGTACGAAC ACATACGTAT GCTAATATGT
-190      -180      -170      -160      -150      -140
GTTTGTATAG TACCCAGTGA TCGCAGACCT GCAATTTT TGTAGGTTTG GAAGAATATA
-130      -120      -110      -100      -90      -80
TAAAGGTTGC ACTCATTCAA GATAGTTTTT TTCTTGTGTG TCTATTCAAT TTATTATGCT
-70      -60      -50      -40      -30      -20
TTGTTTAAAT GTTAAAAAA CCAAGAACTT AGTTTCAAT TAAATTCATC ACAQAAACAA
-10      -1
ACAAAACAAA ATG

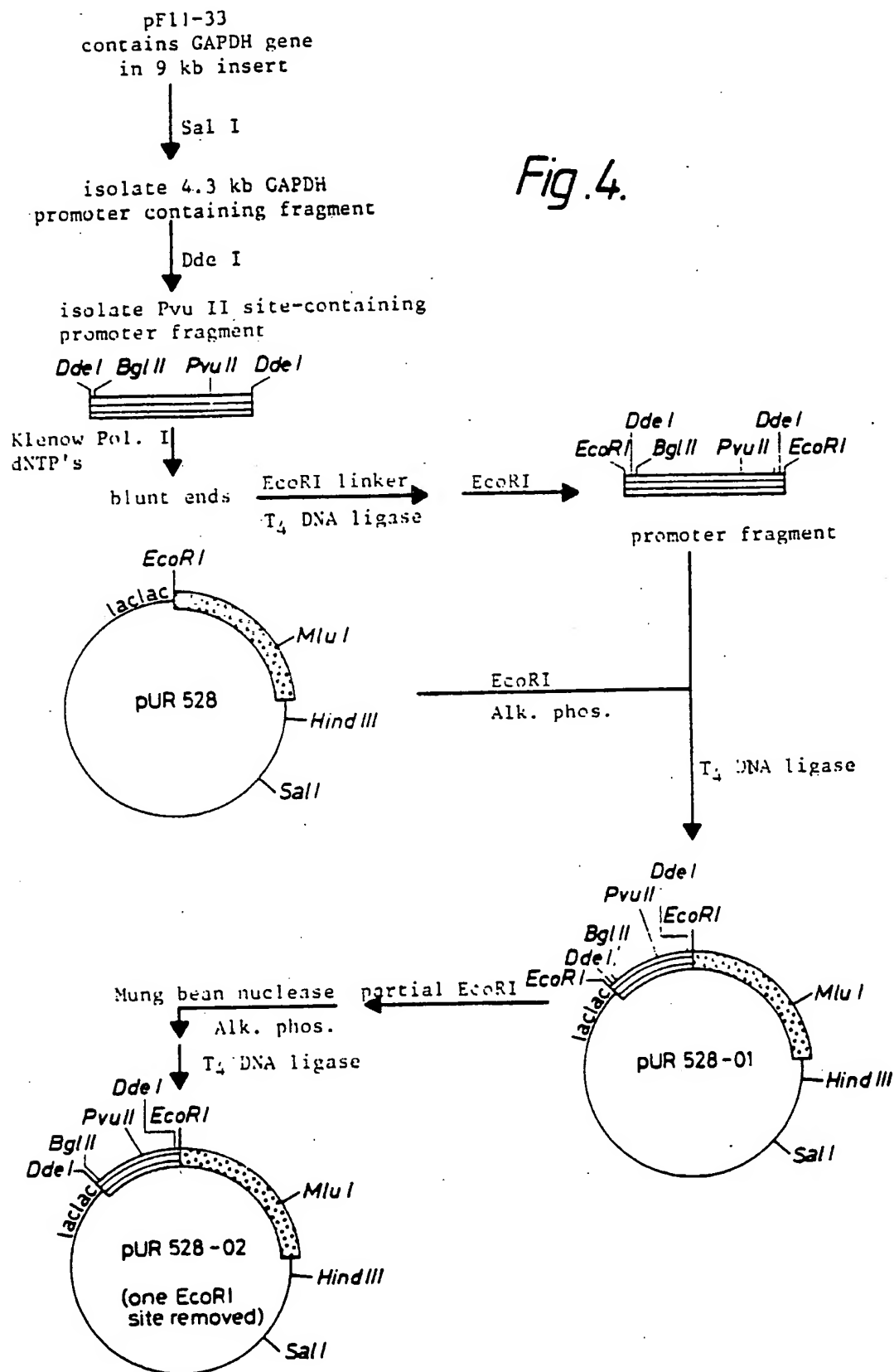
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Fig. 2.

Fig. 3.

7	17	27	37	47	57
TAAATTTAAC	TCCTTAAGGT	TACTTTAATG	ATTTAGTTTT	TATTATTAAT	AATTCATGCT
67	77	87	97	107	117
CATGACATCT	CATATACACG	TTTATAAAAC	TTAAATAGAT	TGAAAATGTA	TTAAAGATTG
127	137	147	157	167	177
CTCAGGGATT	CGATTTTTTT	GGAAGTTTTT	GTTTTTTTTT	CCTTGAGATG	CTGTAGTATT
187	197	207	217	227	237
TGGGAACAAT	TATACAATCG	AAAGATATAT	GCTTACATTC	GACCGTTTTA	GCCGTGATCA
247	257	267	277	287	297
TTATCCTATA	GTAACATAAC	CTGAAGTATA	ACTGACACTA	CTATCATCAA	TACTTGTGAC
307	317	327	337	347	357
ATGAGAATCT	TGTGAATAAT	TAGGCCACTG	AAATTTGATG	CCTGAAGGAC	CGGCATCACG
367	377	387	397	407	417
TATCTTCGAT	AAAGCACTTA	GTATCACACT	AATTGGCTTT	TCGCCGCATA	TGGTGTITTC
427	437	447	457	467	477
GGTGATTTCC	AAGTATTGTT	TCCAAGCATC	GTACCTTTCA	CCATTTGGAG	TATCACTTAG
487	497	507	517	527	537
CGTTTTTCATC	GCATATCTGT	CCATTATTTT	AATGGATTGC	CAAATGGGAA	CTTGATGATG
547	557	567	577	587	597
TGAAAGTTTA	CTCCTAGCAG	TTAACATTTT	CACTTCTGTT	TCCTCTTTAA	TGGCATTTCAT
607	617	627	637	647	657
TCAACTCTTC	CTTGCTTACC	GACGTACCCG	TATATTGGAA	TCTGCGGCCC	CAATGACAGA
667	677	687	697	707	710
AATCACTGCT	TACAATGAAT	AAATTGTTTG	GATCCTTAAT	GTACTCCGAC	AAAATATTAC
727	737	747	757	767	777
CAATGCAACG	ATCAACATCA	ACGCTGTTAT	GAGAAACCAT	CATGGGAATT	ACCTTCACCG
787	797	807	817	827	837
TATCTAAAGA	AATTTCTCTC	CATTTCAAAG	TTTCCACCAA	CATGGGGAGC	TGCATCTCTA
847	857	867	877	887	897
AGGAATGTTT	AGCCATATCA	GTGTCATGAT	CCATTGGCTT	AAACAGCTTC	TTCCGTTCTT
907	917	927	937	947	957
CAGGATACTC	CTTCTGTATT	AATGTTTTAC	ACAAGTCTGT	ATCCACTTTT	AGATTACCCA
967	977	987	997	1007	1017
AGGGCGTCTC	TAGCTCACTG	AATGCACTAA	CTAAAATTTG	GTTTTTGAAA	TAGATGTGAT
1027	1037	1047	1057	1067	1077
GCGACGGCCC	CAAGATAAAT	ATTCTCTTAA	CATTACGGTT	CAAATCCAAC	GATGCGTACG
1087	1097	1107	1117	1127	1137
AGTAGGCCAT	AGTGGGTCCA	CAATACCTGT	AACCGGCATG	AGGACATATG	ATAATTCTGG
1147	1157	1167	1177	1187	1197
CGTTGTGAAT	TGGGCCTTTA	AGGGTACTTT	TGATCAAGTA	TGTATGCGGT	TGTTGAGATA
1207	1217	1227	1237	1247	1257
ATTCTTGGGC	TCTATTGGAA	TACCATGAGC	CTGCATGTGT	TGCTGGACGT	ATTGACATGT
1267	1277	1287	1297	1307	1317
TTGAAAAAAT	CTATTCTTTG	CACTGTAGTC	CACCTAAGCC	ACCGACTAGG	ACCACTTCAC
1322					
TTAAG					

Fig. 4.



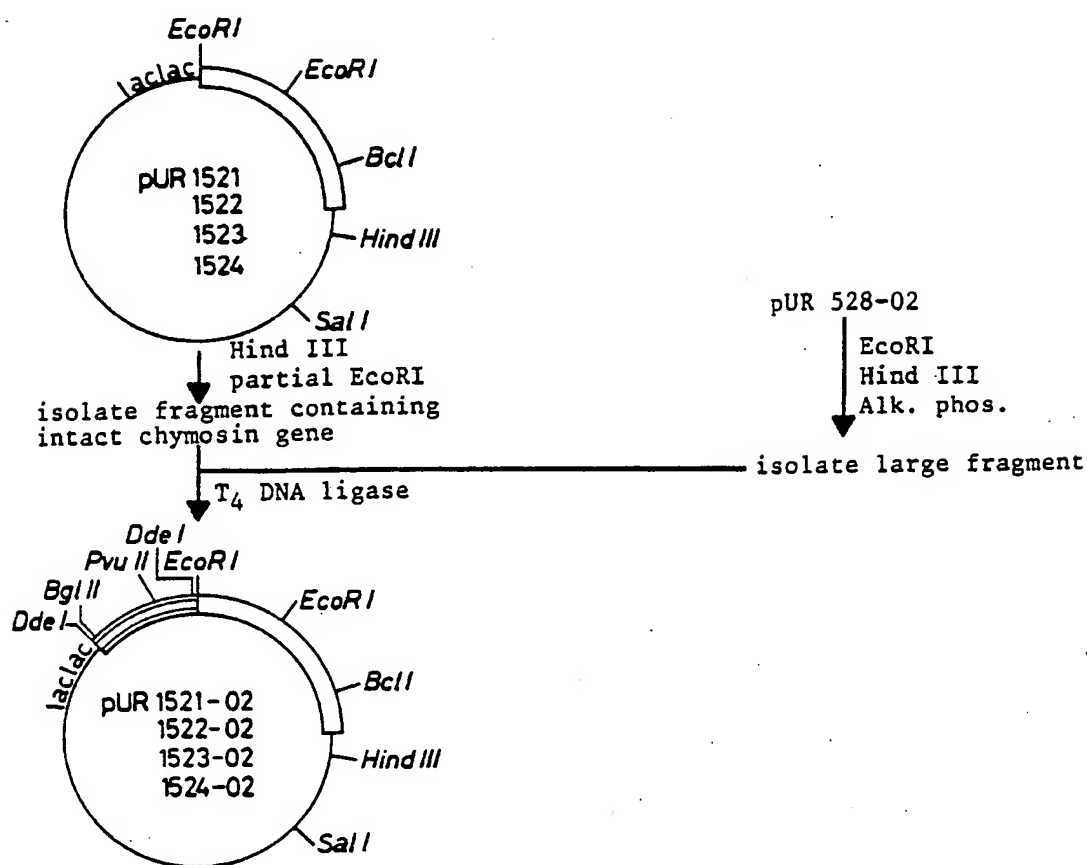


Fig.5.

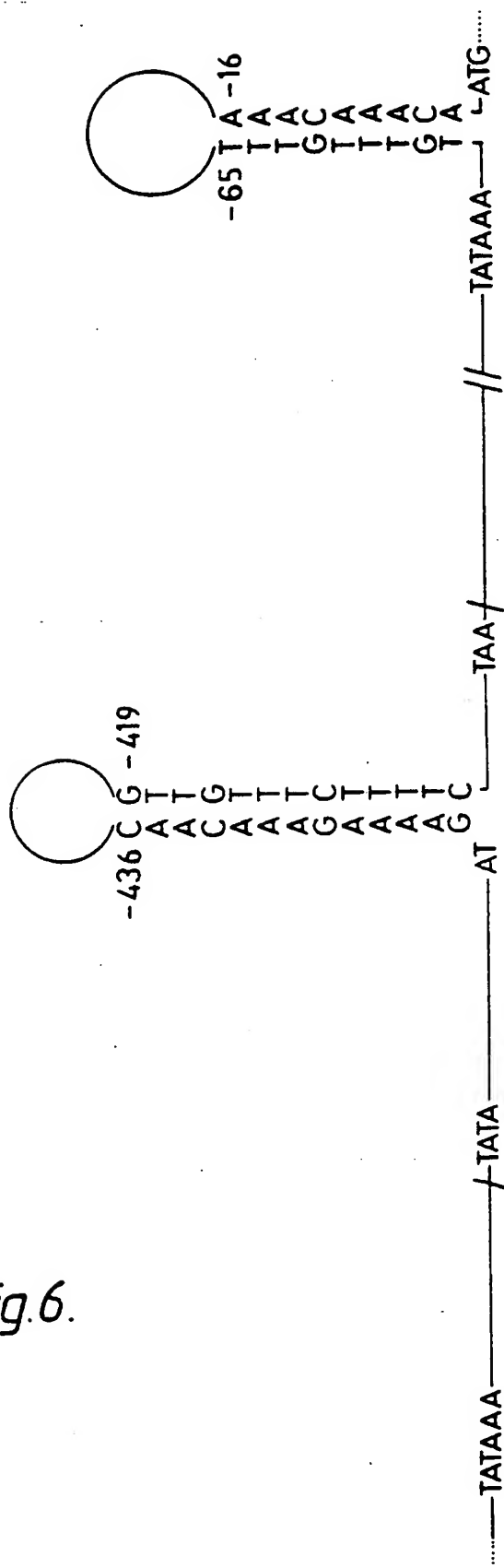


Fig. 6.

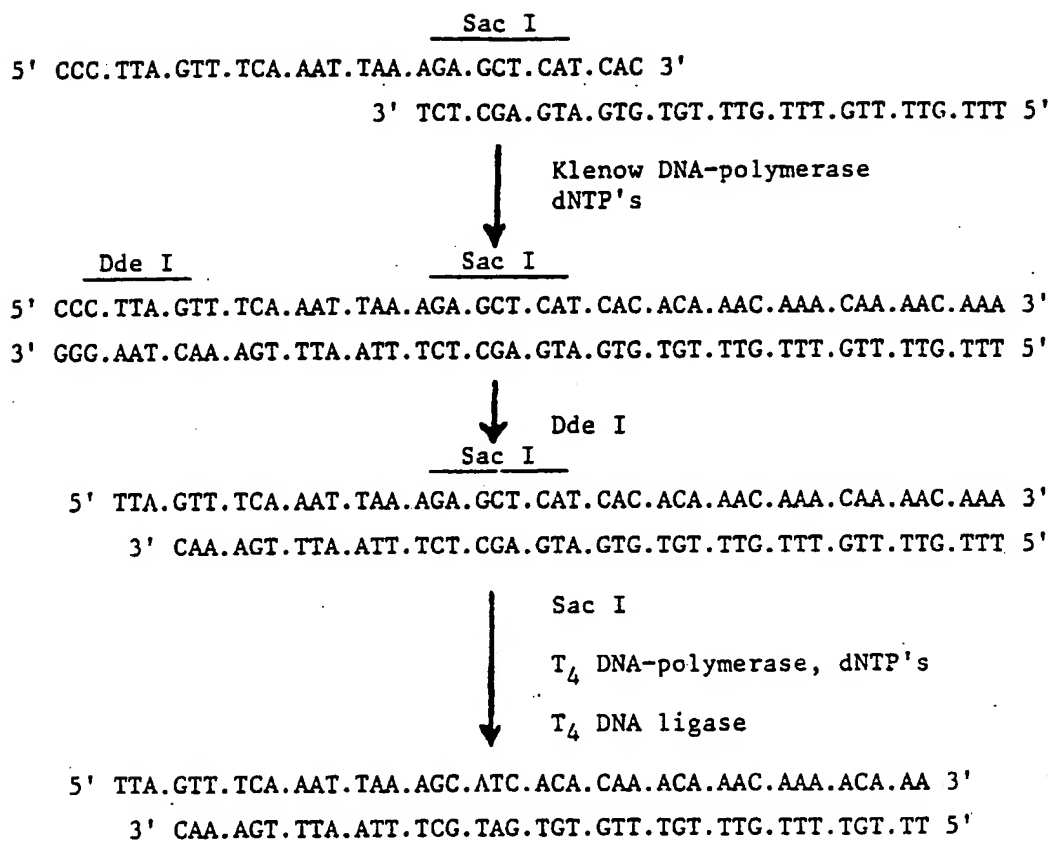
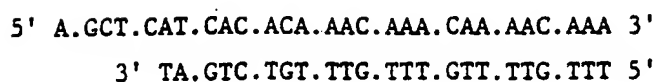
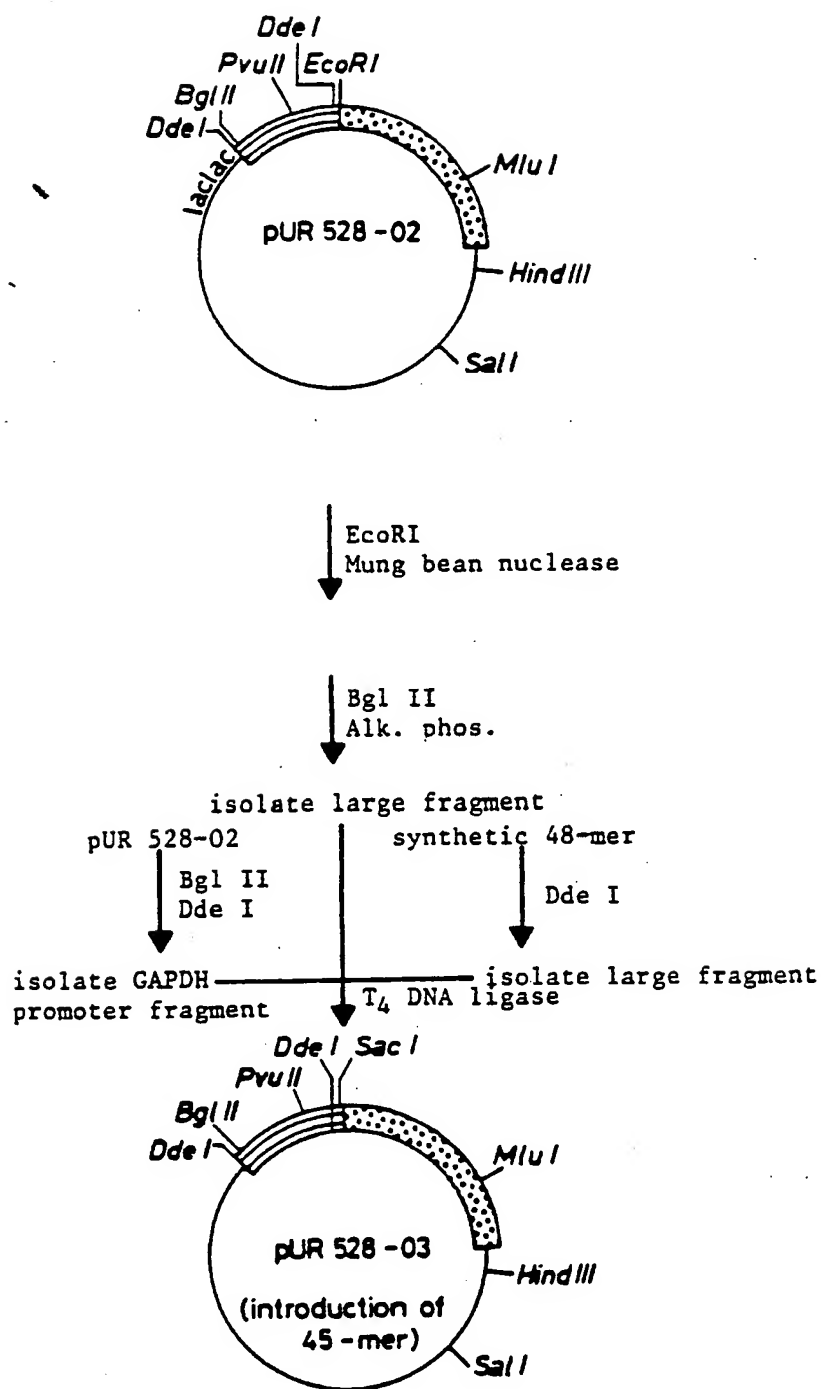
Fig. 7a.*Fig. 7b.*

Fig. 8.



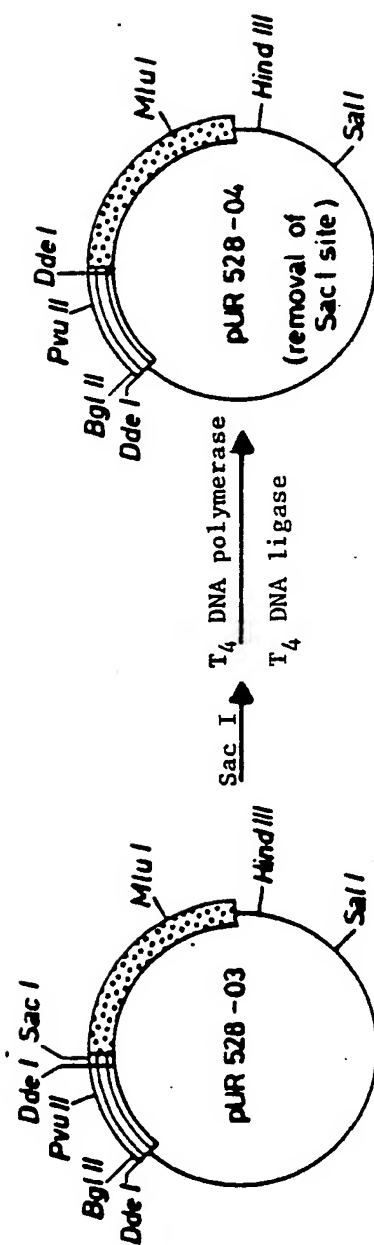


Fig. 9.

Fig.10.

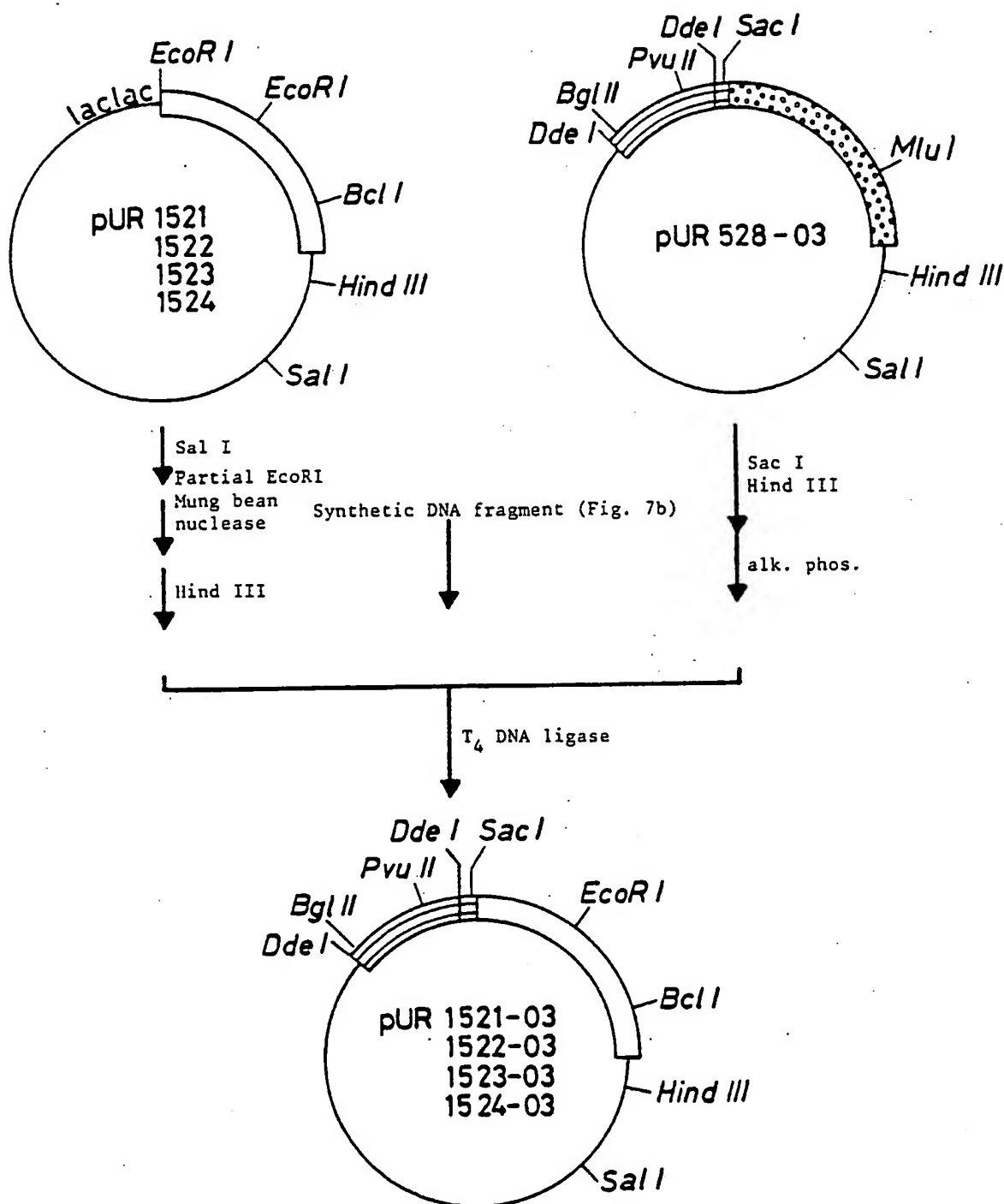
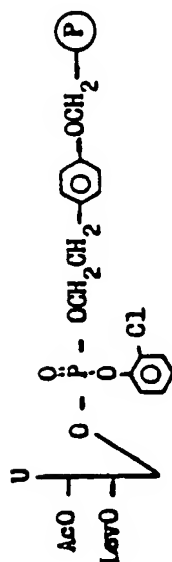
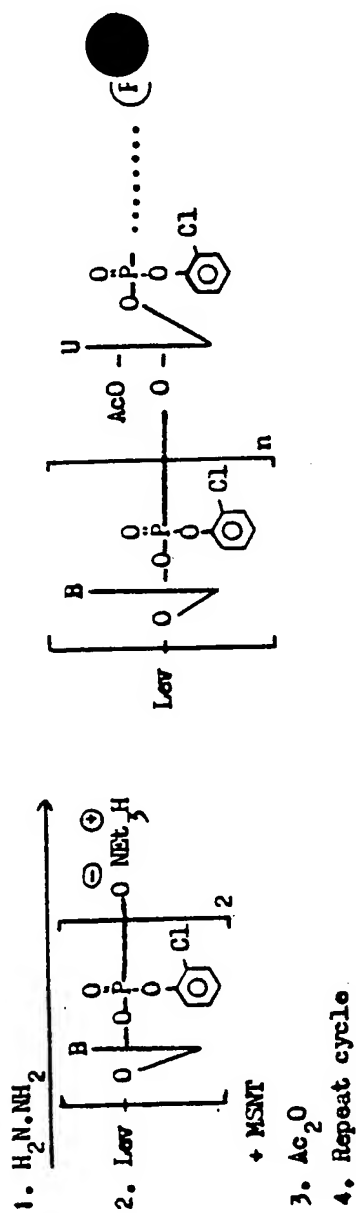


Fig. 11.



B = Bz, C^{An}, DPA, T

Lev = $\text{CH}_3\text{COCH}_2\text{CH}_2\text{CO}$

P = Polystyrene support

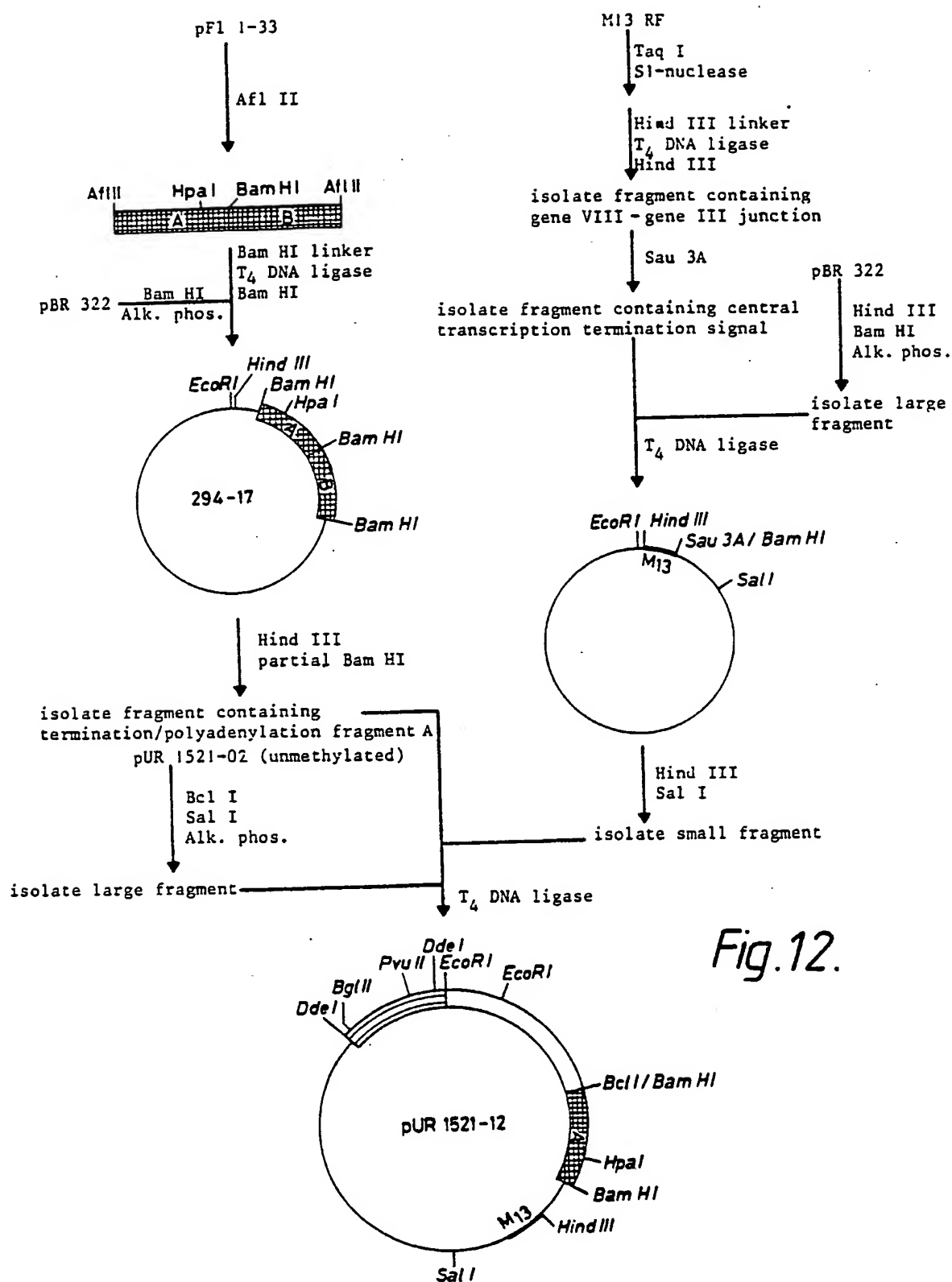


Fig.12.

Fig.13.

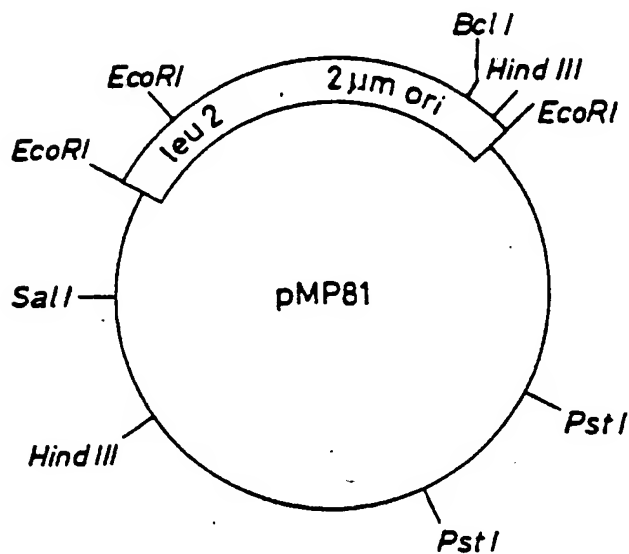


Fig. 14.

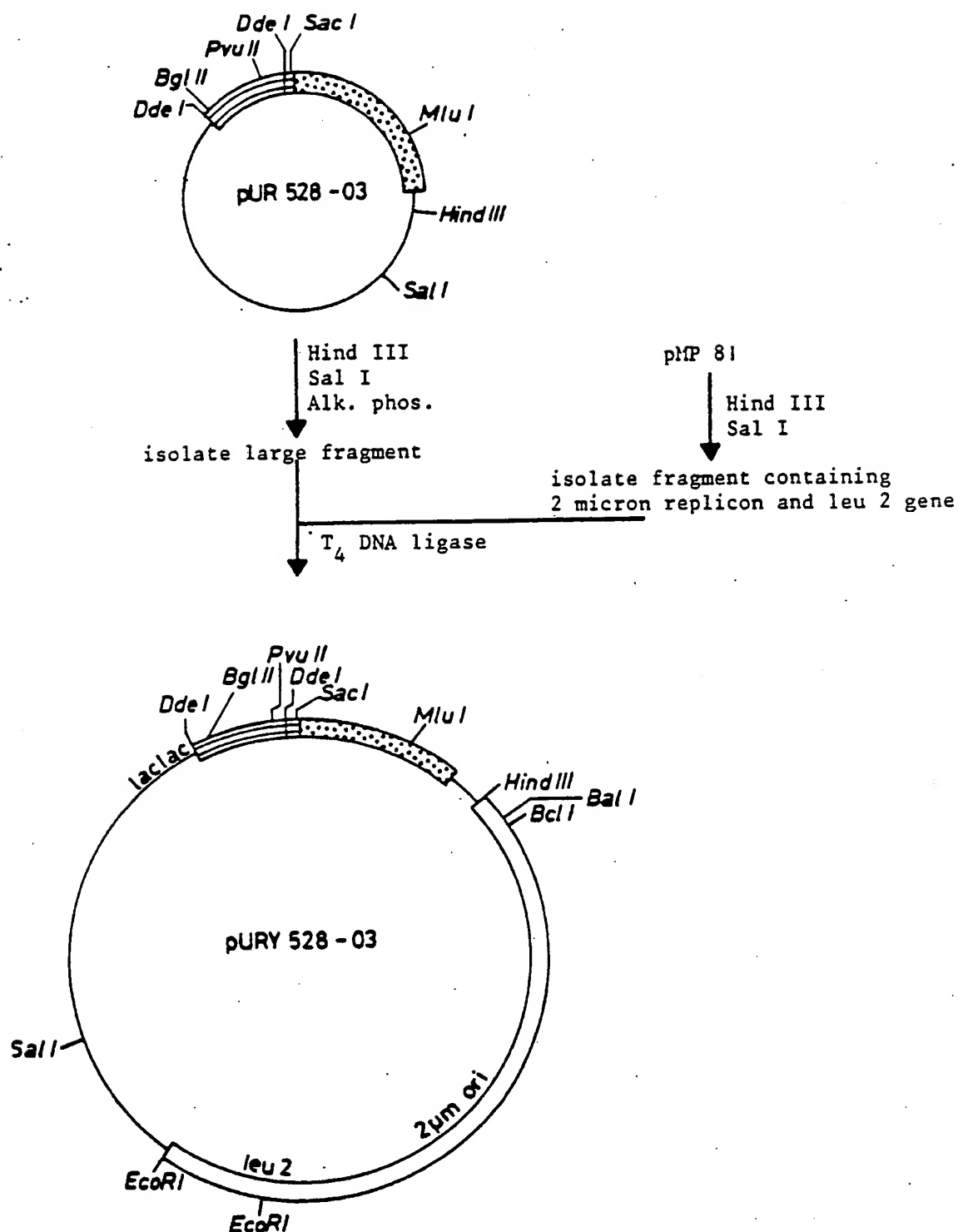


Fig.15.

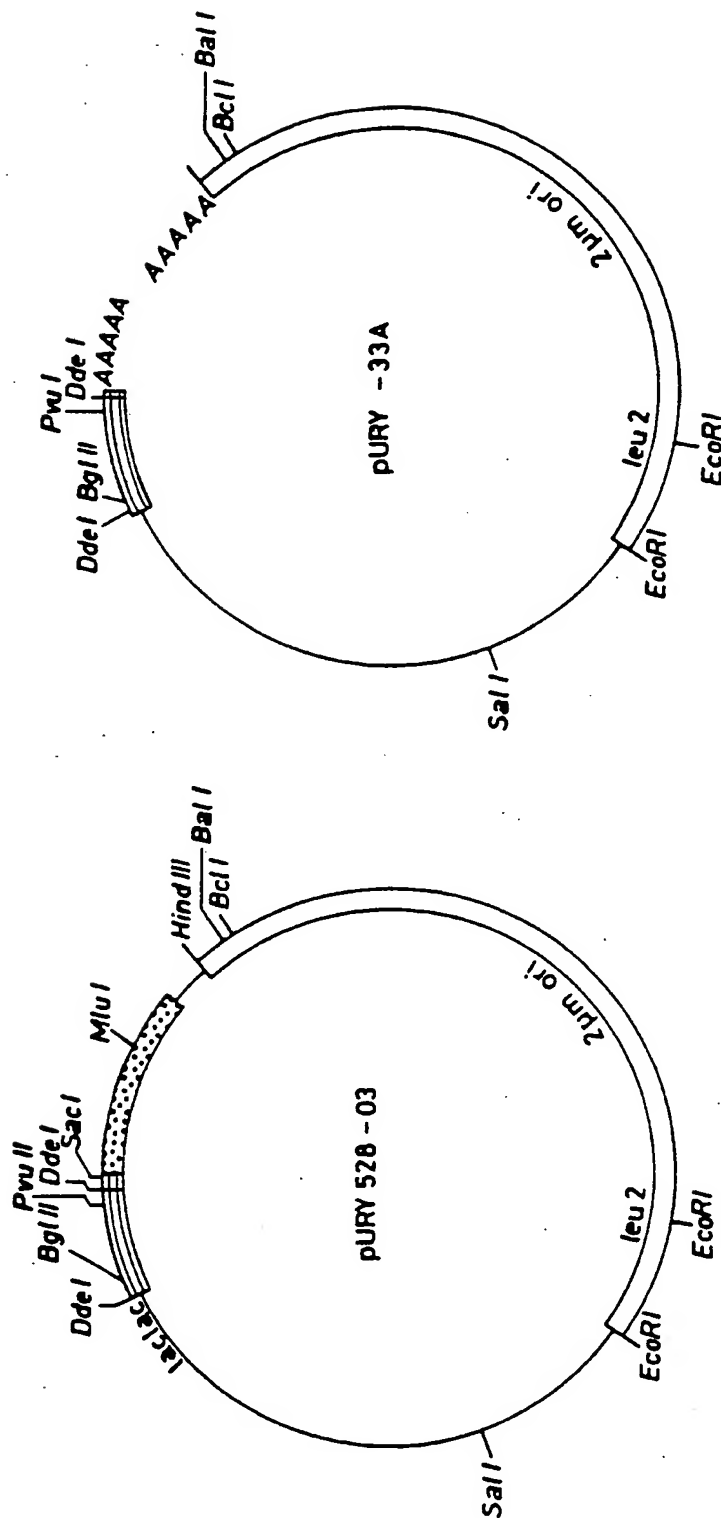


Fig. 16.

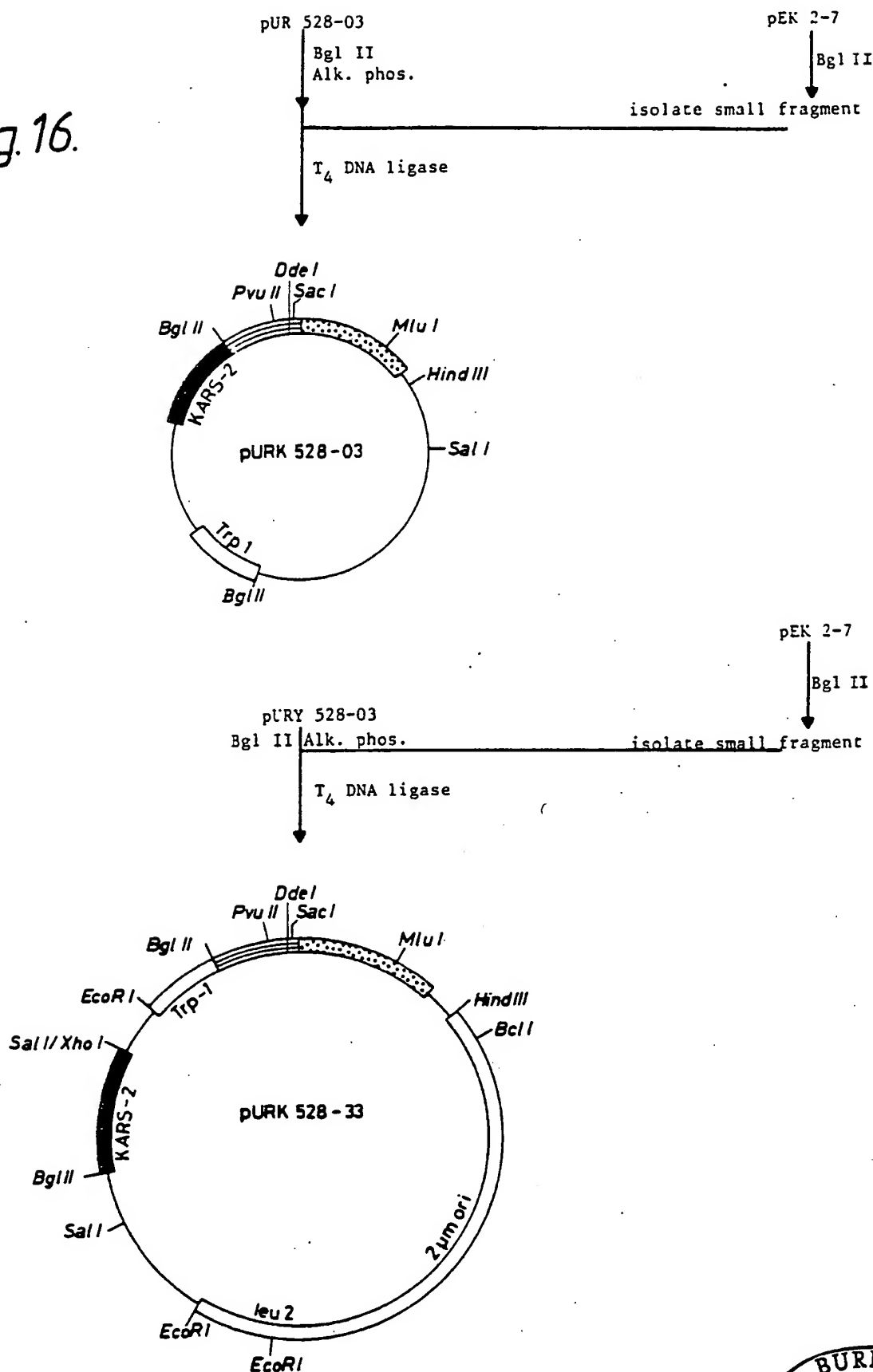
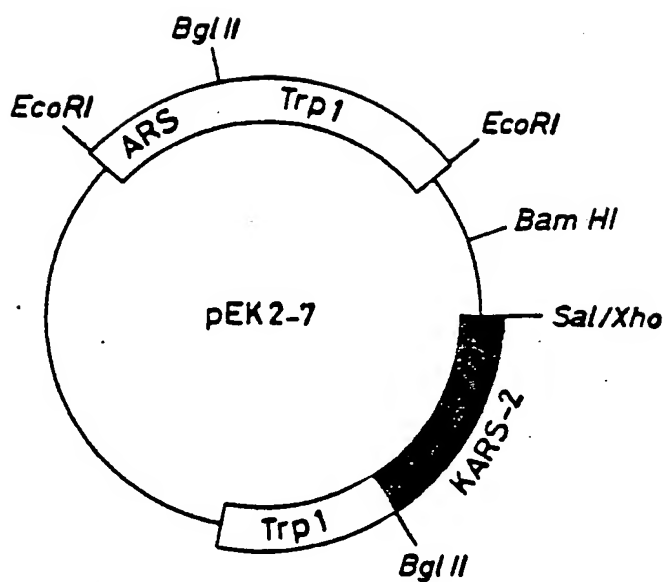


Fig.17



Fig.18.



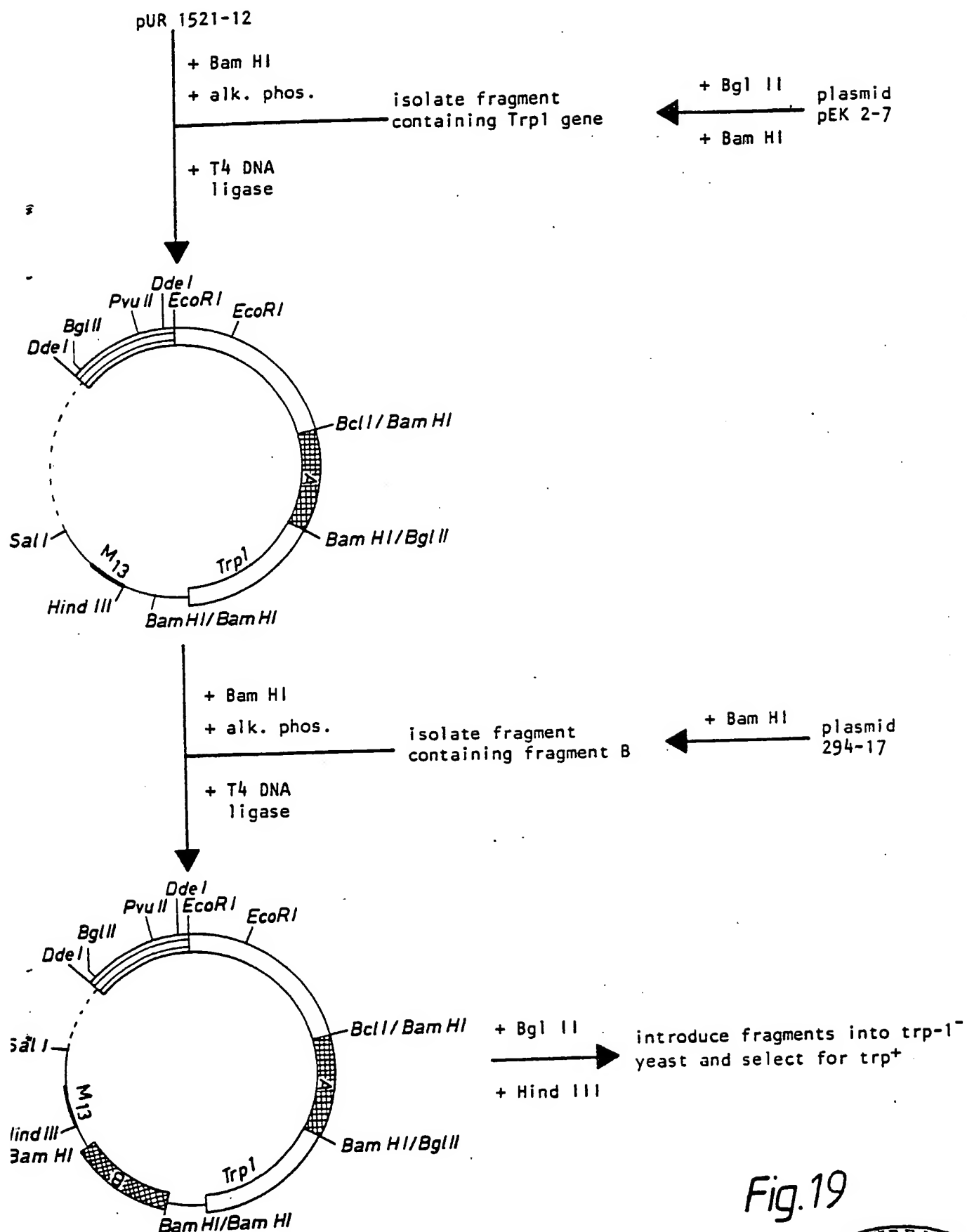


Fig.19

Fig. 20.



Fig. 21.

LEADER SEQUENCE PREPROCHYMOSIN IN S. CER. PREFERRED CODONS

ATGAGATGTT TGGTTGTTTT GTTGGCTGTT TTCGCTTTGT CCCAAGGT
 -174 -165 -155 -145 -135 -127

DNA SEQUENCE PRO PART PRECEEDING PSEUDOCCHYMOSIN IN S. CER. PREFERRED CODONS

GCTGAAATTA CTAGAATTCC ATTGTACAAG GGTAAGTCCT TGAGAAAGGC TTTGAAGGAA
 -126 -116 -106 -96 -86 -76
 CACGGTTTGT TGAAGACTT C
 -66 -56 -46

DNA SEQUENCE PSEUDO PART PRECEEDING CHYMOSIN IN S. CER PREFERRED CODONS

TTGCAAAAGC AACAATACGG TATTTCTCC AAGTACTCCG GTTTC
 -45 -36 -26 -16 -6 -1

DNA SEQUENCE CHYMOSIN IN S. CER. PREFERED CODONS

10	20	30	40	50	60
GGTGAAGTTG	CTTCCGTTCC	ATTGACTAAC	TACTTGGACT	CCCAATACTT	CGGTAAGATT
70	80	90	100	110	120
TACTTGGGTA	CTCCACCACA	AGAATTCAC	GTTTTGTTTC	ACACTGGTTC	CTCCGACTTC
130	140	150	160	170	180
TGGGTTCCAT	CCATTTACTG	TAAGTCCAAC	GCTTGTAAGA	ACCACCAAAG	ATTCGACCCA
190	200	210	220	230	240
AGAAAGTCCT	CCACTTTCCA	AAACTTGGGT	AAGCCATTGT	CCATTCACTA	CGGTACTGGT
250	260	270	280	290	300
TCCATGCAAG	GTATTTTGGG	TTACGACACT	GTTACTGTTT	CCAACATTGT	TGACATTCAA
310	320	330	340	350	360
CAAACGTGTT	GTTTGTCCAC	TCAAGAACCA	GGTGACGTTT	TCACTTACGC	TGAATTCGAC
370	380	390	400	410	420
GGTATTTTGG	GTATGGCTTA	CCCATCCTTG	GCTTCCGAAT	ACTCCATTCC	AGTTTTCGAC
430	440	450	460	470	480
AACATGATGA	ACAGACACTT	GGTTGCTCAA	GACTTGTTC	CCGTTTACAT	GGACAGAAAC
490	500	510	520	530	540
GGTCAAGAAT	CCATGTTGAC	TTTGGGTGCT	ATTGACCCAT	CCTACTACAC	TGGTTCCTTG
550	560	570	580	590	600
CACTGGGTTT	CAGTTACTGT	TCAACAATAC	TGGCAATTCA	CTGTTGACTC	CGTTACTATT
610	620	630	640	650	660
TCCGGTGTGG	TTGTTGCTTG	TGAAGGTGGT	TGTCAAGCTA	TTTTGGACAC	TGGTACTTCC
670	680	690	700	710	720
AAGTTGGTTG	GTCCATCCTC	CGACATTTTG	AACATTCAAC	AAGCTATTGG	TGCTACTCAA
730	740	750	760	770	780
AACCAATACG	GTGAATTGGA	CATTGACTGT	GACAACCTGT	CCTACATGCC	AACGTGTGTT
790	800	810	820	830	840
TTCGAAATTA	ACGGTAAGAT	GTACCCATTG	ACTCCATCCG	CTTACACTTC	CCAAGACCAA
850	860	870	880	890	900
GGTTTCTGTA	CTTCCGGTTT	CCAATCCGAA	AACCACTCCC	AAAAGTGGAT	TTTGGGTGAC
910	920	930	940	950	960
GTTTTCATTA	GAGAATACTA	CTCCGTTTTT	GACAGAGCTA	ACAACTTGGT	TGGTTTGGCT
969					
AAGGCTATT					



Fig. 22.

LEADER SEQUENCE OF PREPROTHAUMATIN IN S.CER. PREFERRED CODONS

ATGGCTGCTA CTACTTGTTT CTTCTTCTTG TTCCCATCTCT TGTGTGTTGT GACTTTGTCC
 -66 -57 -47 -37 -27 -17 -7

AGAGCT
 -1

DNA SEQUENCE MATURE THAUMATIN IN S. CER. PREFERRED CODONS

10	20	30	40	50	60
GCTACTTTTCG	AAATTGTTAA	CAGATGTTCC	TACACTGTCT	GGGCTGCTGC	TTCCAAGGGT
70	80	90	100	110	120
GACGCTGCTT	TGGACGCTGG	TGGTAGACAA	TTGAACTCCG	GTGAATCCTG	GACTATTAAC
130	140	150	160	170	180
GTTGAACCAG	GTACTAAGGG	TGTAAGATT	TGGGCTAGAA	CTGACTGTTA	CTTCGACGAC
190	200	210	220	230	240
TCCGGTAGAG	GTATTTGTAG	AACITGGTGAC	TGTGGTGGTT	TGTTGCAATG	TAAGAGATTG
250	260	270	280	290	300
GGTAGACCAC	CAACTACTTT	GGCTGAATTC	TCCTTGAACC	AATACGGTAA	GGACTACATT
310	320	330	340	350	360
GACATTTCCA	ACATTAAGGG	TTTCAACGTT	CCAATGTACT	TCTCCCCAAC	TACTAGAGGT
370	380	390	400	410	420
TGTAGAGGTG	TTAGATGTGC	TGCTGACATT	GTTGGTCAAT	GTCCAGCTAA	GTTGAAGGCT
430	440	450	460	470	480
CCAGGTGGTG	GTTGTAACGA	CGCTTGTAAT	GTTTTCCAAA	CTTCCGAATA	CTGTTGTAAT
490	500	510	520	530	540
ACTGGTAAGT	GTGGTCCAAC	TGAATACTCC	AGATTCTTCA	AGAGATTGTG	TCCAGACGCT
550	560	570	580	590	600
TTCTCCTACG	TTTTGGACAA	GCCAACTACT	GTTACTTGTC	CAGGTTCTCT	CAACTACAGA
610	620				
GTTACTTTCT	GTCCAACGCT	T			

DNA SEQUENCE ACIDIC PEPTIDE OF PROTHAUMATIN IN S.CER. PREFERRED CODONS

622 632
 TTGGAATTGG AAGACGAA



LEADER SEQUENCE ACIDIC PHOSPHATASE IN YEAST PREFERRED CODONS

ATGTTCAAGT CCGTTGTTTA CTCCATTTTG GCTGCTTCCT TGGCTAACGC T
-51 -41 -31 -21 -11 -1

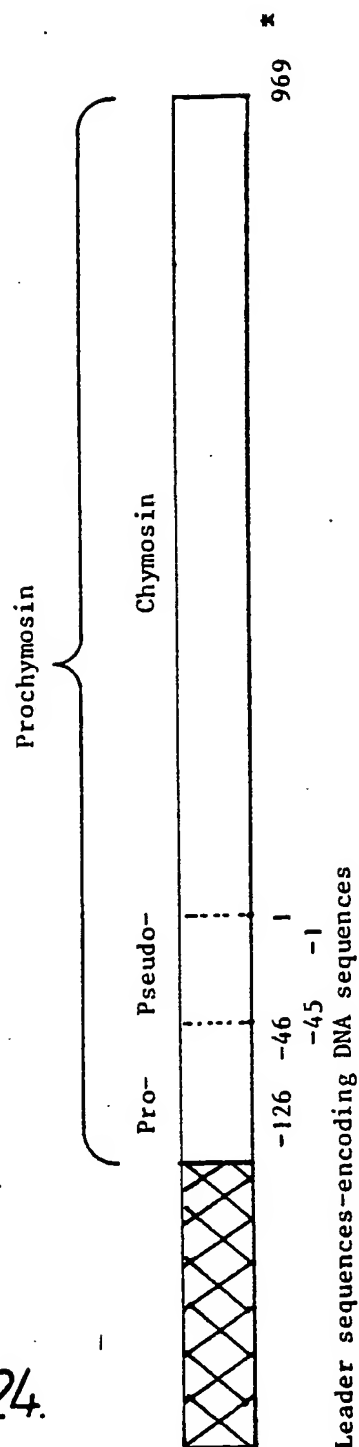
LEADER SEQUENCE INVERTASE IN YEAST PREFERRED CODONS

ATGTTGTTGC AAGCTTTCTT GTTCTTGTTG GCTGGTTTCG CTGCTAAGAT TTCCGCT
-57 -47 -37 -27 -17 -7 -1

Fig. 23.



Fig. 24.



Leader sequences used are those originating from:
 Pre Acid phosphatase (-1 to -51 or -4 to -54*, Fig. 23)
 Pre Invertase (-1 to -54 or -4 to -54, Fig. 23)
 Pre Pro Thaumatin (-1 to -66, Fig. 22)
 and two consensus leader sequences (Fig. 25, -1 to -57).

* nucleotides number according to previous figures.

Fig. 25.

CONSENSUS LEADER SEQUENCE (A-B) IN YEAST PREFERRED CODONS

ATGTCCAAGG CTGCTTTGGC TTTCATTGCT TTCGTTATTG TTTTGATTGT TAACGCT
-48 -38 -28 -18 -8 -1

CONSENSUS LEADER SEQUENCE (B-A) IN YEAST PREFERRED CODONS

ATGTCCAAGT TCGTTATTGT TTGATTGTT GCTGCTTTGG CTTTCATTGC TAACGCT
-48 -38 -28 -18 -8 -1



INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 84/00153

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) *		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ³ : C 12 N 15/00; C 12 P 21/02; C 07 H 21/04; C 12 N 1/16// (C 12 N 1/16; C 12 R 1/645)		
II. FIELDS SEARCHED		
Minimum Documentation Searched *		
Classification System	Classification Symbols	
IPC ³	C 12 N	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category *	Citation of Document, * with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X	EP, A, 0077689 (SUNTORY) 27 April 1983 see pages 3,14,25-36; figure 1; claims 1-8 --	1-3,5,9-12
X	EP, A, 0060057 (GENENTECH.) 15 September 1982 see pages 12,15-22; page 25, lines 19-28; claims 1-14 --	1,3,9-12
X	EP, A, 0073635 (KINGSMAN) 9 March 1983 see claims 1-16; page 4, line 17 --	1,3,9-12
Y	EP, A, 0001931 (GENENTECH.) 16 May 1979 see claims 1-17; figure 2; page 10, lines 15-17, table 1; page 11, lines 1-10 --	7,8
Y	EP, A, 0046039 (SEARLE & CO.) 17 February 1982 see claim 1; page 2, lines 15-18 --	7,8
Y	Drug Development Research 1:435-454 (1981)	./.
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁵</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ³		Date of Mailing of this International Search Report ³
15th October 1984		08 NOV. 1984
International Searching Authority ¹		Signature of Authorized Officer ¹⁰
EUROPEAN PATENT OFFICE		G.L.M. Kruidenberg

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	<p>L. MILLER et al.: "Synthesis of Biologically Active Proteins by Recombinant DNA Technology", pages 435-454 see page 442, lines 7-14</p> <p>--</p> <p>The Journal of Biological Chemistry, vol. 256, no. 3, 10 February 1981 (US) M.J. Holland et al.: "The primary structures of two yeast enolase genes", pages 1385-1395, see page 1389</p> <p>--</p>	<p>7,8</p> <p>4</p>
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹⁰

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:
2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ¹¹

This International Searching Authority found multiple inventions in this international application as follows:

- claims 1,3-12: DNA sequence comprising a DNA sequence according to claim 1, one or more structural genes and at least two other features; Process for introducing these DNA sequences into yeasts; Yeasts containing these DNA sequences; Process for preparing polypeptides by cultivation of these yeasts; and DNA sequence of claim 1 as given in figure 2 °).

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No ¹⁸
Y A	EP, A, 0057350 (COLLABORATIVE RESEARCH) 11 August 1982 see claims 1-40; pages 42-51	4 9-12
	--	
Y	EP, A, 0054330 (UNILEVER) 23 June 1982 see claims 1-8; figure 1	4
E	--	
	EP, A, 0089666 (GENENTECH.) 28 September 1983 see claims 1-12; pages 7,8	1,3,9-12
E	--	
	EP, A, 0096910 (UNILEVER) 28 December 1983 see claims 1-8; figures 1-4	1-5,9-12
E	--	
	EP, A, 0096430 (GIST-BROCADES) 21 December 1983 see claims 1-38; figures 5,6; pages 30-34	1-5,9-12

Continuation of Form PCT/ISA/210 (supplemental sheet (2)), page 2, box VI:		
- <u>claim 2</u> : DNA sequence as given in figure 3		

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO.

PCT/EP 84/00153 (SA 7165)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 01/11/84

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0077689	27/04/83	JP-A- 58146281	31/08/83
EP-A- 0060057	15/09/82	JP-A- 57159489 AU-A- 8065782	01/10/82 02/09/82
EP-A- 0073635	09/03/83	AU-A- 8723882 JP-A- 58077896	24/03/83 11/05/83
EP-A- 0001931	16/05/79	LU-A- 80489 GB-A, B 2007675 NL-A- 7811040 DE-A- 2848051 FR-A- 2422717 FR-A, B 2422718 FR-A- 2422719 JP-A- 54092696 AU-A- 4136178 OA-A- 6002 US-A- 4356270 AU-B- 527165 SE-A- 7811460 AT-B- 373281	22/03/79 23/05/79 10/05/79 10/05/79 09/11/79 09/11/79 09/11/79 23/07/79 17/05/79 30/06/81 26/10/82 17/02/83 09/05/79 10/01/84
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For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82

INTERNATIONAL APPLICATION NO.
-----PCT/EP 84/00153 (SA 7165)

EP-A- 0096430	21/12/83	EP-A-	0096910	28/12/83
		WO-A-	8304050	24/11/83
		WO-A-	8304051	24/11/83
		AU-A-	1553983	02/12/83

For more details about this annex :
see Official Journal of the European Patent Office, No. 12/82
